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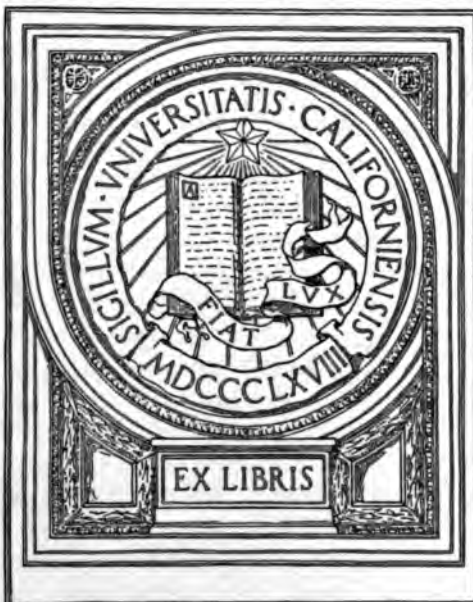


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CREATINURIA IN WOMEN.

By MARY S. ROSE.

CORRECTIONS.

On page 538, Vol. XXXI, No. 3, September, 1917, the 5th line from the bottom, for *This, however, does disprove Scott's contention* read *This, however, does not disprove Scott's contention*.

On page 613, for

Lyxohexosamine (synthetic chondrosamine)

↓

Lyxohexosaminic acid (synthetic)

read

Lyxohexosamine (synthetic chondrosamine)

↑

Lyxohexosaminic acid (synthetic)

On page 619, 20th line, for *60 gm.* read *6.0 gm.*

On page 645, over the heading of the second column of the tabulation for $C_{69}H_{203}NO_{12}$ read $C_{69}H_{105}NO_{12}$.

causes a prompt appearance of creatine in the urine, always accompanied

¹ Rose, W. C., *J. Biol. Chem.*, 1911-12, x, 265. Folin, O., and Denis, W., *ibid.*, 1912, xi, 253.

² Krause, R. A., *Quart. J. Exp. Physiol.*, 1914, vii, 87.

³ Krause, *Quart. J. Exp. Physiol.*, 1911, iv, 293.

⁴ Underhill, F. P., *J. Biol. Chem.*, 1916, xxvii, 127.

⁵ McCollum, E. V., and Steenbock, H., *J. Biol. Chem.*, 1912-13, xiii, 209.



CREATINURIA IN WOMEN.

By MARY S. ROSE.

(From the Department of Nutrition, Teachers College, Columbia University,
New York.)

(Received for publication, August 9, 1917.)

Normal men do not excrete creatine in appreciable amounts on a diet which is free from preformed creatine and creatinine, or on a diet containing creatine unless the amounts are large or the diet very high in protein. Normal children, however, excrete endogenous creatine,¹ and according to Krause,² the younger the child the less its ability to retain creatine administered *per os*. Normal women exhibit an intermittent creatinuria. Krause³ kept under observation for several months a group of women suffering from disabilities which did not interfere with metabolism, and although the diet was creatine- and creatinine-free, creatine was always present after menstruation, but in most cases absent or present in mere traces 2 or 3 weeks later. It sometimes persisted through 4 weeks, especially if menstruation were irregular.

The significance of the appearance of endogenous creatine in the urine is obscure. Current views have recently been discussed by Underhill,⁴ who points out the well recognized association of the elimination of creatine with perversions of carbohydrate metabolism, and also calls attention to the fact that carbohydrate deficiency does not explain all the cases in which creatine appears in the urine. Thus creatinuria in the pig on a diet ample in carbohydrate was attributed by McCollum and Steenbock⁵ to the character of the proteins in the diet, while Underhill and his associates have pointed out that "in nearly every instance in which creatine appears in the urine there is an accompanying acidosis," and have demonstrated experimentally that in the case of the rabbit a diet of oats and corn causes a prompt appearance of creatine in the urine, always accompanied

¹ Rose, W. C., *J. Biol. Chem.*, 1911-12, x, 265. Folin, O., and Denis, W., *ibid.*, 1912, xi, 253.

² Krause, R. A., *Quart. J. Exp. Physiol.*, 1914, vii, 87.

³ Krause, *Quart. J. Exp. Physiol.*, 1911, iv, 293.

⁴ Underhill, F. P., *J. Biol. Chem.*, 1916, xxvii, 127.

⁵ McCollum, E. V., and Steenbock, H., *J. Biol. Chem.*, 1912-13, xiii, 209.

Daily Elimination of Creatine and creatinine.

I			II			III			IV			V			VI			VII			VIII		
Date.	Creatinine.	Creatinine.	Date.	Creatinine.	Creatinine.	Date.	Creatinine.	Creatinine.	Date.	Creatinine.	Creatinine.	Date.	Creatinine.	Creatinine.	Date.	Creatinine.	Creatinine.	Date.	Creatinine.	Creatinine.	Date.	Creatinine.	Creatinine.
1915	mg.	mg.	1916	mg.	mg.	1915	mg.	mg.	1916	mg.	mg.	1917	mg.	mg.	1917	mg.	mg.	1916	mg.	mg.	1917	mg.	mg.
Feb. 20	197	80	Mar. 25	352	48	Feb. 17	583	0	Mar. 27	459	0	Feb. 20	235	47	Feb. 23	583	7	Mar. 7	427	0	Feb. 23	291	15
" 21	108	36	" 26	" 18	514	94	" 28	368	57	" 21	536	19	" 24*	530	0	" 8	302	0	" 24	257	8
" 22	94	37	" 27	266	242	" 19	572	12	" 29	381	85	" 22	291	8	" 25*	583	0	" 9	543	0	" 25	338	4
" 23	85	63	" 28	423	53	" 20	572	24	" 30	323	53	" 23*	519	0	" 26*	716	0	" 10	558	0	" 26	286	0
" 24	129	18	" 29	397	86	" 21	397	140	" 31	380	51	" 24*	346	0	" 27	580	11	" 11	334	0	" 27	310	0
" 25	130	20	" 30	376	148	" 22	584	125	Apr. 1	" 25*	485	0	" 28	675	18	" 12	431	0	" 28	558	0
" 26	130	20	" 31	471	71	" 23	531	118	" 2	499	18	" 26	348	0	Mar. 1	551	56	" 13*	561	0	Mar. 1	357	0
" 27	135	24	Apr. 1	" 24	413	32	" 3	316	27	" 27	314	0	" 2	653	9	" 14*	387	0	" 2
" 28	147	22	" 2	431	164	" 25	411	80	" 4	256	147	" 28	233	0	" 3	575	73	" 15*	442	0	" 3	470	51
Mar. 1	162	52	" 3	366	37	" 26	523	81	" 5*	414	0	Mar. 1	323	0	" 4	568	19	" 16	294	91	" 4	288	3
" 2	177	93	" 4	400	94	" 27	375	135	" 6*	469	0	" 2	333	0	" 5	570	25	" 17	367	6	" 5*	416	26
" 3	281	87	" 5	278	71	" 28	428	69	" 7*	391	25	" 3	268	0	" 6	" 18	276	26	" 6*	369	0
" 4	356	136	" 6	427	50	Mar. 1	456	62	" 8	" 4	286	0	" 7	476	70	" 19	127	0	" 7*	379	0
" 5	356	136	" 7	272	104	" 2	555	164	" 9	" 5	313	0	" 8	636	6	" 20	162	19	" 8	279	0
" 6*	325	68	" 8	242	101	" 3	536	75	" 10	503	104	" 6	276	0	" 9	568	0	" 21	181	35	" 9	395	12
" 7*	264	53	" 9	" 4	488	110	" 11	466	60	" 7	374	52	" 10	589	50	" 22	155	90	" 10	451	28
" 8*	261	60	" 10	344	149	" 5	487	0	" 12	529	96	" 8	385	0	" 11	590	33	" 23	203	110	" 11	464	34
" 9	256	61	" 11*	321	24	" 6	497	5	" 13	512	81	" 9	355	0	" 12	597	38	" 24	240	0	" 12
" 10	234	44	" 12*	282	36	" 7*	491	0	" 14	491	43	" 10	223	0	" 13	642	6	" 25	" 25

" 11	222	39	" 13*	" 8*	" 15	" 15	" 11	" 0	" 14	" 0	" 26	" 0
" 12	215	44	" 14	" 9*	" 16	" 16	" 12	" 0	" 15	" 20	" 27	" 103
" 13	259	47	" 15	" 10	" 17	" 17	" 13	" 0	" 16	" 8	" 28	" 0
" 14	" 16	" 11	" 18	" 18	" 14	" 0	" 17	"	" 29	" 149
" 15	283	58	" 17	" 12	" 19	" 19	" 15	" 0	"	"	" 30	" 80
" 16	390	31	" 18	" 13	" 20	" 20	" 15	" 0	"	"	" 31	" 94
" 17	364	85	" 19	" 14	" 21	" 21	" 15	" 0	"	"	Apr. 1	"
" 18	389	52	" 20	" 15	" 22	" 22	" 15	" 0	"	"	" 2	"
											" 3	" 110
											" 4	" 44
											" 5	" 109
											" 6	" 105
											" 7	" 92
Diet uniform throughout, N intake 6.7 gm. (average) per day.												
Diet free from preformed creatine and creatinine but not otherwise controlled.												
Diet fairly uniform throughout, N intake 20 or more gm. per day, largely as skim milk powder.												
Mar. 7-17 potatoes and butter only; thereafter mixed diet free from preformed creatine and creatinine.												
Diet Feb. 23 - Mar. 5 mixed but free from preformed creatine and creatinine; Mar. 6-11, potatoes and butter only.												

* Menstruation.

† Reported as creatinine.

by acidosis, as measured by the hydrogen ion content of the urine. That the proteins of the diet were without special significance in this connection seems equally well proven.

Underhill and Baumann⁶ have also shown that in phlorhizin glycosuria creatinuria occurs even when large quantities of alkali are administered, and that hypoglycemia resulting from injection of hydrazine sulfate is accompanied by an elimination of creatine. Thus it seems clear that "creatinine elimination in the urine may be induced by at least two sets of conditions: (1) creatine may appear in the urine where carbohydrate deficiency is not involved and (2) creatinuria may be present during carbohydrate deficiency even in the absence of acidosis."

That the amount of protein in the diet may be a factor is suggested by Denis,⁷ who finds that forced protein feeding induces creatinuria in certain cases of hyperthyroidism, whereas a low protein diet produces a creatine-free urine. She has also reported similar results⁸ from varying the protein intake of children. None of these findings seems to explain the intermittent creatinuria of women.

From time to time studies have been made in this laboratory which it was hoped would throw further light on this problem, and eight of them, made upon six normal women, are here reported. On account of the erratic character of the creatine elimination as shown by preliminary trials, short periods were without significance. Daily estimations of creatinine and creatine were therefore continued from 18 to 32 days, creatinine being determined by Folin's method and creatine by Benedict's modification. The data of the individual experiments are given in the table.

The creatine output varied greatly with the individual. As may be seen from the table, in some instances it appeared every day for which determinations were made (I and II), while in others it was detected only occasionally (VI and VIII). The subject of I served also a year later for II, and similarly III and IV represent the same individual. There is more resemblance between two curves for the same person than between any two from different sources, but aside from the tendency of these two persons to excrete creatine almost continuously there is no striking uniformity. One point of interest was to see whether any quantitative relationship existed between the creatine and creatinine, and it will be noted that very often when the creatine was high

⁶ Underhill, F. P., and Baumann, E. J., *J. Biol. Chem.*, 1916, xxvii, 151.

⁷ Denis, W., *J. Biol. Chem.*, 1917, xxx, 47.

⁸ Denis, W., and Kramer, J. G., *J. Biol. Chem.*, 1917, xxx, 189.

the creatinine was low, and *vice versa*, but in plotted curves the sum of the two did not make a straighter line than either one alone. In six out of the eight experiments creatine disappeared with the onset of menstruation, and reappeared shortly afterward, with one exception (V).

The diet was in all cases free from preformed creatine and creatinine. In I, where creatine appeared constantly, but in varying amounts, the diet was weighed, analyzed, and kept uniform from day to day. For other purposes urine and feces were analyzed, and the nitrogen intake ranged from 6 to 7 gm. per day for a subject weighing 60 kilos. In this case a relatively high protein diet was certainly not the cause of the creatinuria. There was no lack of carbohydrate and the diet was specially chosen to avoid acidosis. A year later on a diet less carefully controlled as to quantity and variety, there was a constant creatinuria (II). Subject VII was on a diet consisting exclusively of potatoes and clarified butter for the first 10 days, and no creatine appeared. The diet was then changed to a mixed one, free from creatine and creatinine, and creatine appeared and disappeared at irregular intervals during the remainder of the experiment. Another subject was then put upon a potato diet (VIII) after having been for 10 days under observation on a mixed but creatine- and creatinine-free ration. This subject was able to take the potato diet only a week, but in the 6 days reported, creatine was excreted half the time. It hardly seems likely that the potato diet had any specific influence, though this point needs further study.

There is no correlation between diets high in protein and the creatine output in these cases. Subjects V and VI were for other purposes on a diet containing from 20 to 25 gm. of nitrogen per day, the protein furnishing fully 25 per cent of the total fuel value of the ration and derived very largely from skim milk powder. Yet V excreted creatine less often than any other subject, while VI exhibited an irregular but quantitatively low creatine elimination. Again, Subjects I and VII were on diets very low in protein, and I showed a constant creatinuria while VII eliminated almost no creatine when the protein content of the diet was the lowest.

These experiments do not throw any light on the ultimate cause

of the creatinuria, but they seem to the writer to uphold the view of Krause² in regard to the explanation of the difficulty with which children destroy administered creatine, and of Benedict and Osterberg³ from their observations of the way in which phlorhizinized dogs react to this substance, that creatine is constantly being formed in relatively large amounts and is normally for the most part either utilized or destroyed; that the adult man can metabolize considerable amounts, while the child's powers are much weaker, so that creatine in the latter represents a balance between formation and destruction; and that women occupy a position intermediate between men and children, being able to metabolize most if not all of the creatine which they may produce, the amount fluctuating with perhaps a variety of conditions.

CONCLUSIONS.

The creatine output of women is very irregular, and no definite relationship between creatine and creatinine has been demonstrated.

There is no clearly defined connection between the creatine output and the sexual cycle. Creatine tends to disappear at the onset of menstruation and to reappear shortly afterwards, but it may do the same at other times also.

Creatine is excreted on diets liberal in carbohydrate and causing no acidosis; it is not definitely influenced by the amount of protein in the diet.

² Benedict, S. R., and Osterberg, E., *J. Biol. Chem.*, 1914, xviii, 195.

METABOLISM OF FATS.

I. UTILIZATION OF PALMITIC ACID, GLYCERYL PALMITATE, AND ETHYL PALMITATE BY THE DOG.

By J. F. LYMAN.

(From the Laboratory of Agricultural Chemistry, Ohio State University, Columbus, and the Sheffield Laboratory of Physiological Chemistry, Yale University, New Haven.)

(Received for publication, July 16, 1917.)

It was found by Frank¹ that ethyl palmitate fed to dogs appears in the thoracic lymph as the glyceryl ester. The most probable source of the glycerol required for this transformation is carbohydrate. To test this hypothesis Professor Lafayette B. Mendel suggested to me that Frank's experiments be repeated using dogs made completely diabetic by phlorhizin, on the theory that such animals might have lost, wholly or partly, the capacity to convert carbohydrate into glycerol because of a perverted sugar metabolism. When the attempt was made, even with normal animals, the amount of fat recovered from the chyle was so small that the whole plan appeared futile and suggested that ethyl palmitate was very slowly and perhaps poorly absorbed. Accordingly it was decided to compare the utilization of ethyl palmitate with that of the glyceryl ester and of the free acid.

Experiments to determine the utilization of relatively pure free fatty acids and their esters are not numerous in the literature. It seems desirable that further information be obtained as a basis for a better understanding of fat metabolism.

The following data on this subject have been published.

¹ Frank, O., *Z. Biol.*, 1898, xxxvi, 568.

TABLE I.
Utilization of Various Fatty Acids and Esters by Dogs.

Material.	Utilisation.	Experimenter.
	<i>per cent</i>	
Stearic acid.....	35-53	Levites. ²
Palmitic acid.....	67-78	"
Oleic ".....	91-98	"
Na stearate.....	87	"
" palmitate.....	90	"
" oleate.....	100	"
Tristearin.....	9-14	Arnschink. ³
Ethyl palmitate.....	75	Frank. ¹
" stearate.....	12	"
Cetyl palmitate.....	51-69	Munk. ⁴
Amyl oleate.....	Nearly complete.	"

Palmitic acid was prepared from bayberry tallow.⁵ Palmitic acid and ethyl alcohol were esterified by passing dry HCl gas into the warm mixture. The product melted at about 24° and contained less than 0.1 per cent free palmitic acid. It had a Koettstorfer number of 198, theoretical for ethyl palmitate = 197.6, and absorbed 0.2 per cent of iodine by the Wijs procedure. Glyceryl palmitate was prepared by heating 36 gm. of glycerol and 100 gm. of palmitic acid at 160-180° for 16 hours with constant stirring, cooling, and repeated washing with hot water. The product contained 8.3 per cent free palmitic acid, 91 per cent total palmitic acid, free and combined, and melted at about 60°.

The dogs were fed daily a basal ration of 200 gm. of lean meat from which the visible fat had been removed, 50 gm. of cracker crumbs, and 10 gm. of agar. To this basal ration 40 gm. of the fat under investigation were added (in the case of glyceryl palmitate 34 gm.) and the whole was thoroughly mixed with 350 cc. of boiling water. In this manner even the hard fats were melted and well incorporated. The experimental periods were of 4 days' duration and were separated by a 4 day (in some cases

² Levites, S., *Z. physiol. Chem.*, 1900, iii, 349.

³ Arnschink, L., *Z. Biol.*, 1890, xxvi, 434.

⁴ Munk, I., *Arch. Anat. u. Physiol.*, 1890, 581.

⁵ Chittenden, R. H., and Smith, H. E., *Am. Chem. J.*, 1884-85, vi, 217.

a 3 day) rest period during which 40 gm. of lard were added to the basal ration. The feces were marked off with finely ground cork, collected daily, and kept at a low temperature until the last feces from the diet period were obtained. The feces were then weighed, mixed by passing through a hashing machine three times, and the fatty acids contained in them at once determined by the Gephart and Csonka⁶ method. The stools were well formed in all cases except during the ethyl palmitate period, when they were somewhat soft. In calculating the utilization values allowance was made for the fatty acids eliminated in the feces when the diet consisted of the basal ration plus an amount of carbohydrate isodynamic with 40 gm. of fat.

Dog I, Male, Weight 11.8 Kg.

Length of period, days	Fat fed.	Fatty acids fed per day.	Average weight of feces per day.	Fatty acids in feces, average.	Fatty acids in feces per day.	Utilized.		
		gm.	gm.	per cent	gm.	gm.	per cent	Corrected. per cent
4	Lard.	38	49	3.75	1.83	36.17	95.2	96.6
4	Ethyl palmitate.	36.5	116	13.41	15.56	20.44	57.4	58.8
4	Lard.	38	58	2.15	1.25	36.75	96.7	98.1
4	Glyceryl palmitate.	31	41.5	5.12	2.13	28.87	93.1	94.8
3	Lard.	38	54	4.52	2.66	35.34	93.0	94.4
4	Palmitic acid.	40	61.5	12.24	7.53	32.47	81.2	82.5
3	Lard.	38	68.0	2.3	1.47	36.53	96.1	97.5
4	Fat-free diet (?)		62.5	0.87	0.53			

Dog II, Male, Weight 12.8 Kg.

4	Lard.	38	46.8	4.67	2.18	35.82	94.3	96.2
4	Ethyl palmitate.	36.5	125.4	14.8	18.66	17.84	48.9	50.9
4	Lard.	38	48.5	2.27	1.35	36.65	96.4	98.4
4	Glyceryl palmitate.	31	39	5.52	2.15	28.85	93.1	95.4
3	Lard.	38	46	5.69	2.73	35.27	92.9	94.8
4	Palmitic acid.	40	70.6	11.82	8.35	31.65	79.1	81.0
3	Lard.	38	54.5	3.75	2.03	35.97	94.7	96.5
4	Fat-free diet (?)		38	1.95	0.74			

⁶ Gephart, F. C., and Csonka, F. A., *J. Biol. Chem.*, 1914, xix, 521.

The average of all the trials shows a utilization for lard of 96.6 per cent, a value which agrees closely with the results of Langworthy and Holmes⁷ and of Smith, Miller, and Hawk⁸ who found respectively 97 and 96.8 per cent utilization for pork fat. This is good evidence that our animals were normal as regards the utilization of fats and that the method of study employed was satisfactory. Ethyl palmitate, although liquid at body temperature, was utilized to the extent of only 50.9 and 58.8 per cent in the two trials, a value considerably lower than that obtained by Frank.¹ The feces from Dog I during this period contained 15 per cent ether extract, consisting of 20.6 per cent free fatty acids calculated as palmitic acid, and 79.4 per cent neutral fat, which was identified by its melting point as ethyl palmitate. Fatty acids combined as soaps were present in the feces to the extent of 0.9 per cent. These facts indicate that absorption of unhydrolyzed ethyl palmitate does not readily occur. Further, the fact that normal feces contain 75 to 90 per cent of the eliminated fatty acids free or combined as soaps⁹ while ethyl palmitate is recovered in the feces largely unchanged indicates that hydrolysis of the ethyl ester in the body is slow. Our experiment supports the contention of Terroine¹⁰ that absorption of fats is limited by the rate of hydrolysis and not *vice versa*. Further, our experiments by the lymph fistula method support the view held by Bloor¹¹ that emulsified neutral fats cannot be absorbed as such.

It seems worthy of note that ethyl stearate is very poorly absorbed as compared with ethyl palmitate—10 and 50 per cent respectively. Somewhat the same relationship holds between the utilization of the glyceryl esters of these two acids, *viz.*, 12 and 95 per cent. Considerable emphasis has been put on the melting point of fats as a determining factor for digestibility. It appears to the author that the case is not so simple as this, for while the corresponding esters of palmitic and stearic acids do

⁷ Langworthy, C. F., and Holmes, A. D., *U. S. Dept. Agric., Office of Home Economics, Bull. 310*, 1915.

⁸ Smith, C. A., Miller, R. J., and Hawk, P. B., *J. Biol. Chem.*, 1915, xxiii, 505.

⁹ Munk, *Arch. Path. Anat. u. Physiol.*, 1884, xcv, 454.

¹⁰ Terroine, É. F., *J. de Physiol.*, 1911, 695.

¹¹ Bloor, W. R., *J. Biol. Chem.*, 1913, xv, 105.

not differ markedly as to melting points, the ethyl esters of both being liquids and the glyceryl esters being solids at body temperature, still there is a wide difference as to digestibility. We suggest that the nature of the fatty acid radicle of an ester has an effect on digestibility aside from its effect on the melting point of the compound. Experiments are in progress to determine this point.

The objection may be raised to our conclusion that hydrolysis is a prerequisite to absorption of fats, *viz.*, that if such is true then palmitic acid should be better utilized than glyceryl palmitate, when as a matter of fact the opposite is found to be true. It seems to us that there is a simple explanation which brings the hypothesis into harmony with the facts. Fatty acids when fed in large amounts are irritating to the digestive tract, causing the food mass to be discharged slower from the stomach,¹² probably increasing peristalsis, and certainly increasing the volume of feces. In other words, feeding large amounts of free fatty acids disturbs the mechanics of digestion.

SUMMARY.

With two dogs the following utilization values were obtained: lard, 96.7 and 96.5 per cent, ethyl palmitate 58.8 and 50.9 per cent, glyceryl palmitate 94.8 and 95.4, palmitic acid 82.5 and 81.0.

Emulsified esters of fatty acids are not absorbed as such, but absorption is limited by the rate of hydrolysis.

It is suggested that the melting point of the ester is not the only factor, probably not the chief factor, determining the rate of hydrolysis and absorption.

¹² Frank, *Arch. Physiol.*, 1894, 297.

METABOLISM OF FATS.

II. THE EFFECT OF FEEDING FREE PALMITIC ACID, GLYCERYL PALMITATE, AND ETHYL PALMITATE ON THE DEPOT FAT IN THE WHITE RAT.

By J. F. LYMAN.

(From the Laboratory of Agricultural Chemistry, Ohio State University, Columbus, and the Sheffield Laboratory of Physiological Chemistry, Yale University, New Haven.)

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That depot fat in the animal is influenced in texture and iodine absorption number by the character of the food fat has been amply demonstrated by the experiments of Hofmann,¹ Munk,² Lummert,³ Rosenfeld,⁴ and others. Cell fat, on the contrary, is constant in character and is not affected by the diet.⁵ From Bloor's⁶ work it appears that the character of fats is modified during their absorption, so that hard fats become softer and soft fats harder; i.e., there is a tendency toward constancy in the character of the fat stores of the animal. Free fatty acids of the food are recovered in the chyle almost entirely as neutral fat.⁷ Monoglycerides during absorption are changed to triglycerides.⁸ After feeding cetyl palmitate,⁹ amyl oleate,¹⁰ or ethyl palmitate,¹¹ the corresponding triglycerides only can be found in the thoracic lymph. There appears, then, to be a tendency for

¹ Hofmann, F., *Z. Biol.*, 1872, viii, 153.

² Munk, I., *Arch. Anat. u. Physiol.*, 1884, xcv, 407.

³ Lummert, W., *Arch. ges. Physiol.*, 1898, lxxi, 176.

⁴ Rosenfeld, G., *Verhandl. Kong. inn. Med.*, 1898, xvii, 503.

⁵ Abderhalden, E., and Brahm, C., *Z. physiol. Chem.*, 1910, lxxv, 330.

⁶ Bloor, W. R., *J. Biol. Chem.*, 1914, xvi, 517.

⁷ Walther, P., *Arch. Anat. u. Physiol.*, 1890, 329.

⁸ Argyris, A., and Frank, O., *Z. Biol.*, 1912, lix, 143.

⁹ Munk, I., and Rosenstein, A., *Arch. path. Anat. u. Physiol.*, 1891, cxxiii, 230.

¹⁰ Munk, *Arch. Physiol.*, 1890, 581.

¹¹ Frank, O., *Z. Biol.*, 1898, xxxvi, 568.

triglycerides during absorption to pass over into the chyle and fat depots unchanged or with slight modification in melting point and degree of saturation, while all other esters and free fatty acids are converted into the corresponding triglycerides during the process of absorption.

The proof that esters of the fatty acids other than triglycerides are unabsorbed as such is not complete, however; for in all the experiments only a small part of the ester fed has been recovered in the thoracic lymph, while the effect on depot fat has not been studied. There remains then the possibility of the absorption of unchanged ester, *e.g.*, ethyl palmitate, by way of the portal vein.¹² In order to get a somewhat broader view of fat metabolism it was planned to study the character of the fat deposited in the fat depots of animals as a result of feeding free palmitic acid, glyceryl palmitate, and ethyl palmitate. It was planned further to determine the effect of phlorhizin poisoning in each case; but the amounts of fat stored by the phlorhizinized animals were so small that we regard that part of the experiment of too slight value for discussion here. It is hoped to attack the problem of glycerol synthesis in the animal body in some more satisfactory way.

Adult white rats were used as experimental animals. The food consisted of a basal ration of commercial dog biscuit (which had been finely ground and extracted with ether), casein, and dried yeast. To this the fat under investigation was added to make an equivalent of 30 per cent palmitic acid; *e.g.*, the palmitic acid diet contained 30 per cent palmitic acid, 40 per cent dog biscuit, 28 per cent casein, and 2 per cent yeast. The ethyl palmitate diet contained 33.3 per cent ethyl palmitate, 36.6 per cent dog biscuit, 28 per cent casein, and 2 per cent yeast. The control diet was made up as follows: 70 per cent dog biscuit, 28 per cent casein, and 2 per cent yeast. This mixture was moistened with 1 per cent solution of Liebig's meat extract and baked into a cake which could be eaten without scattering. The fat-containing diets were made into cakes by heating the mixtures above the melting point of the fat involved, and cooling. Palmitic acid and its esters were prepared according to the

¹² Hamburger, H. J., *Arch. Physiol.*, 1900, 554.

methods described in the preceding paper. Two animals were fed on each diet. They were fasted until 25 per cent loss in body weight had occurred; then the feeding period began, and continued as long as the gain in weight was satisfactory, usually about 2 weeks. The depot fat was dissected from the carcass and, together with the hide, was extracted with a mixture of three parts 95 per cent alcohol and one part ether until practically all the fat had been removed. The intestine, bones, and muscular and glandular tissues were discarded entirely. The alcohol-ether solution was evaporated to dryness under reduced pressure in an atmosphere of carbon dioxide. The residue was taken up in dry pure ether, filtered, and the ether removed by evaporation. The fat from the two animals on the same diet was combined. Iodine absorption number was determined by Wijs' method; glycerol by the acetin process.¹³

The fatty acids were prepared by saponifying the fats with alcoholic sodium hydroxide, extracting the soap solution with ether to remove unsaponifiable matter, liberating the fatty acids with phosphoric acid, shaking out with ether, and drying in a vacuum desiccator.

The table shows the results.

Rat No.	Weight of rat.		Weight of fat.	M.P.	I No.	Free acids as palmitic.	Koettstorfer No.	Glycerol.	Fatty acids.			Diet.
	Initial.	Final.							M.P.	I No.	Mean mol. wt.	
	gm.	gm.	gm.	°C.		per cent		per cent	°C.			
13	196	243	15.4	44	53.9	1.0	206	10.6	47	56.2	258	Ethyl palmitate.
18	238	244	15.6									
19	250	245	8.0	41	41.8	2.1	207	11.1	43	40.5	250	Palmitic acid.
20	228	238	7.0									
6	273	310	26.7	41	48.7	1.3	203	11.8	43	50.4	251	Glyceryl palmitate.
8	203	257	17.3									
17	143	172	11.0	Below 15	70.3	1.1	198	10.3	42	71.4	261	Fat-free.
15	221	264	16.0									

¹³ Lewkowitsch, J., *The Chemical Technology of Oils, Fats, and Waxes*, London, 5th edition, 1915, i.

The fat deposited in the fat depots after feeding free palmitic acid, glyceryl palmitate, or ethyl palmitate appears to consist largely of tripalmitin in all cases, although unsaturated fatty acids are present as shown by the iodine absorption values. The Koettstorfer number of pure tripalmitin is 208.8; that of the fat deposited in these experiments ranges from 203 to 207. Tripalmitin yields 11.42 per cent of glycerol; while that obtained in these experiments amounted to 10.6 to 11.8 per cent of the fat. The molecular weight of tripalmitin is 256; while the mean molecular weight of the fatty acids here obtained was 250 to 258. There is no indication that appreciable amounts of ethyl palmitate are deposited in the fat depots after feeding the ethyl ester; nor is the free fatty acid content of the depot fat considerably raised by feeding free palmitic acid. On a fat-poor diet the depot fat differs markedly from that laid down as a result of feeding palmitic acid or its esters. This fat, which must have been derived chiefly from carbohydrates and proteins, contained more of the unsaturated fatty acids of molecular weight higher than palmitic acid.

SUMMARY.

After feeding free palmitic acid, glyceryl palmitate, or ethyl palmitate to white rats, essentially the same kind of fat is stored in the fat depots, and it consists largely of tripalmitin.

Neither free palmitic acid nor ethyl palmitate in appreciable amounts is deposited unchanged in the fat depots.

The fat deposited as a result of feeding a fat-poor diet differs markedly from that laid down when the diets contain palmitic acid or its esters.

The author wishes to express his gratitude to Professor Lafayette B. Mendel for laboratory space and equipment, and for the aid and advice extended during the experimental work recorded in this and the preceding paper.

THE DETERMINATION OF FECAL INDOLE.

· BY OLAF BERGEIM.

(From the Laboratories of Physiological Chemistry of Jefferson Medical College, Philadelphia, and the University of Illinois, Urbana.)

(Received for publication, August 22, 1917.)

Of the large number of color reactions for indole which have been discovered only the β -naphthaquinone sodium monosulfonate reaction of Herter and Foster¹ has so far given much promise as a basis for the accurate determination of indole in feces. The reaction is one of the most delicate known for indole (a matter of great importance in view of the minute amounts of indole present in the feces under many circumstances), appears to be of high specificity (not reacting with skatole which almost constantly accompanies indole in the feces and from which it is otherwise very difficult to separate it), and is found to develop quantitatively without subjection to arbitrary conditions not readily complied with. Hence the belief expressed by Herter and Foster that their method would prove more accurate than any in use for the determination of indole and that it would prove of service in fecal analysis appears justified.

It therefore seems somewhat unfortunate that this method as applied to feces was not developed in detail and supported by experimental data. As far as we are aware only Gorter and de Graaff² have attempted to use this method. They claim it to be trustworthy and found that the reaction was more delicate than claimed by the original investigators. They do not, however, supply the deficiencies noted. We have had occasion to carry out a large number of determinations of fecal indole and to develop this method in detail and with certain modifications. The

¹ Herter, C. A., *J. Exp. Med.*, 1905, vii, 79. Herter, C. A., and Foster, M. L., *J. Biol. Chem.*, 1905-06, i, 257; 1906-07, ii, 267.

² Gorter, E., and de Graaff, W. C., *Compt. rend. Soc. biol.*, 1908, lxi, 402. Gorter, E., *Arch. méd. enf.*, 1908, xi, 593.

procedure we have found most satisfactory is given below with some observations upon its accuracy and limitations.

Method.

The feces are distilled from alkaline solution to remove phenols. This distillate is again distilled from acid solution to remove ammonia (or the ammonia removed with exchange silicate). The final distillate is treated with β -naphthaquinone sodium monosulfonate and alkali, and the blue indole compound formed extracted with chloroform and determined colorimetrically.

EXPERIMENTAL.

Rub 30 to 50 gm. of the fresh, well mixed feces in a mortar with water to a uniform consistency. Transfer to a wide-mouthed Kjeldahl flask of about 1,000 cc. capacity, rinsing the mortar and the neck of the flask with distilled water to make about 400 cc. Add 5 cc. of 10 per cent KOH solution and about 2 cc. of paraffin to decrease foaming. Distill with steam, using the ordinary Kjeldahl distillation apparatus with a good stream of water in the condenser. Heat carefully for a few minutes until danger of foaming is past and then allow to boil vigorously. Distill over 500 cc. of liquid, bringing the volume of the fecal suspension down to about 100 cc. toward the end of the distillation. Then proceed according either to *a* or *b*, as follows.

a. Transfer the distillate to a clean Kjeldahl flask, and add 2 drops of phenolphthalein as an indicator. Make neutral with *N* sulfuric acid and add 1 cc. excess. Distill with steam as before, collecting the first 500 cc. of distillate and bringing the residue finally to about 100 cc. Mix the distillate well by shaking.

b. Transfer 100 cc. of the first distillate to a 300 cc. Erlenmeyer flask. Add 20 gm. of "Permutit" exchange silicate according to Folin and Bell.³ Rotate moderately for 5 minutes. Pour off, washing the residue with a little water.

Into a 150 cc. conical separatory funnel introduce an aliquot (100 cc.) of distillate from *a* or the supernatant fluid from *b*. Add 1 cc. of a 2 per cent solution of β -naphthaquinone sodium

³ Folin, O., and Bell, R. D., *J. Biol. Chem.*, 1917, xxix, 329.

monosulfonate solution and then 2 cc. of 10 per cent KOH. Shake and let stand for 15 minutes. Extract with chloroform, shaking vigorously, using a 10 cc. and a 7 cc. portion, which will bring the total volume of the extract nearly to the mark of a 15 cc. graduated cylinder. Make exactly to mark and mix well.

Run at the same time and in the same way a standard using 1 cc. of a solution of indole, 0.1 mg. indole per cc. Compare the extract with this standard in a colorimeter, using the standard ordinarily at the 30 mm. mark. Calculate the indole to the basis of mg. of indole per gm. of moist feces.

DISCUSSION.

Indole is carried over quantitatively under the conditions of distillation. In eight determinations in which as much as 40 gm. of feces of high solid content were used, extra 100 cc. portions of distillate from both the acid and alkaline distillations were taken in separate flasks and tested for indole by the usual method. These tests showed that if, in distilling, the fecal suspension is brought down to about 100 cc. toward the end of the process only the very slightest tests for indole were obtained in any case, while in the acid distillation it was not necessary to boil as low as this. Where, however, the volume of the mixture was maintained at over 200 cc. throughout, appreciable amounts of indole were found in the second distillates. With still larger amounts of feces it may be necessary to carry distillation further. In case of doubt it is easy to collect extra 100 cc. portions of distillate separately. These can be tested and indole determined directly without acid distillation as all ammonia will previously have been carried off.

The amount of alkali used in distillation is sufficient to hold back phenols without causing excessive frothing. In the acid distillation the phenolphthalein and sulfuric acid are not volatile with steam while the small excess of acid used decreases the difficulty of carrying over indole and lessens the danger of loss through decomposition.

In running a number of determinations at one time it is desirable to have a common source of steam with regulatory valves at each flask. Such an apparatus may be readily improvised

from a large round-bottomed flask or boiler, some Y-tubes, and Hoffman clamps. Regulation of the distillations does not offer much difficulty.

In the development of the reaction the use of lesser amounts of alkali and especially of the naphthaquinone compound caused undue delay especially where fecal distillates were employed. This appears to outweigh the objection of Herter and Foster that the pale yellow color of the naphthaquinone modifies the pink color of the chloroform solution. This latter interferes very little where as much as 0.1 mg. of indole is present. With much smaller amounts it must be considered. As in most colorimetric work it is, however, desirable to have about the same amounts of indole in unknown and standard.

We have tested the use of amyl alcohol as a substitute for chloroform in part or wholly, and find that while comparable results are obtained as well as a color (wine-pink) more distinct from that of the naphthaquinone itself than in the case of chloroform, there is a tendency for a slight opacity to develop when used for fecal distillates which renders the use of amyl alcohol less desirable for ordinary purposes of fecal analysis.

Standard indole solutions are preferably made up fresh although they may be kept for some days in the ice box without noticeable change. They should be discarded if a yellow tinge develops. The naphthaquinone solution should be made up every few days and kept in the ice box. When a residue forms in the flask the solution should be discarded. If fecal distillates are not to be used at once they should be placed in the ice box, particularly in hot weather.

The 15 minute period allowed for the indole naphthaquinone reaction is sufficient and a much longer time is not desirable as the color weakens.

Indole determinations on feces should be made with the fresh material. In some instances, at least, an appreciable amount of indole is lost on even 1 day's standing in the ice box. It is possible that this loss might be prevented in some way but this problem has been insufficiently studied.

It is of course desirable to avoid the necessity for double distillation. This may be accomplished as indicated above by the use of exchange silicate as suggested by Folin and Bell for the

No.	Sample No.	Amount of feces tak- en.	Indole per gm. of moist mat- ter.
		gm.	mg.
1	I	40	0.0259
2		40	0.0262
3	0.02 mg. of indole per gm. added.	40	0.0459
4	0.02 " " " " " "	40	0.0456
5	II	40	0.0175
6		40	0.0180
7	III	40	0.0138
8		30	0.0140
9	IV	28	0.0151
10		30	0.0152
11	V	40	0.0135
12		40	0.0139
13	VI	30	0.0240
14		30	0.0242
15	VII	40	0.0351
16		40	0.0357
17	VIII	40	0.0341
18	36 hours in ice box.	40	0.0278
19	IX	40	0.0195
20	7 days in ice box.	40	0.0010
21	X	40	0.0151
22	9 days in ice box.	40	0.0119
23	XI	40	0.0110
24	Reagent 1 week on shelf.	40	0.0092
25	Standard solution 1 week on shelf (0.1 mg.).		0.083
26	Standard and reagent 1 week on shelf.		0.070
27	Indole 0.5 mg.; ammonia 20 mg. Modification b.		0.502
28	Same as No. 27 but no ammonia.		0.500
29	" " " 27 " with distillation.		0.507

determination of urinary ammonia. The ammonia is removed and the indole remains behind in solution. We have not used this modification as extensively as the other but in a number of cases identical results were obtained. Inasmuch as the feces may possibly, in certain cases, contain interfering substances other than ammonia passing over in alkaline distillation both procedures are given.

The table shows the agreement of duplicate determinations as carried out by this method and the quantitative recovery of added indole. Determinations 11 to 16 represent the first estimations of a second observer. Determinations 17 to 22 illustrate the effect of standing on the indole content of feces, showing the losses to be pronounced but variable. Numbers 23 to 26 show deterioration of reagents on standing at room temperature. Numbers 27 to 29 indicate that the exchange silicate removes all interfering ammonia but removes no indole.

We consider the modification of the Herter-Foster procedure as described in this paper to be a more accurate method for the determination of fecal indole than any hitherto proposed.

The author is indebted to Dr. H. R. Fishback for assistance in carrying out this work.

SOME ASPECTS OF THE TEMPERATURE COEFFICIENTS OF LIFE PROCESSES.

By W. J. V. OSTERHOUT.

(From the Laboratory of Plant Physiology, Harvard University, Cambridge.)

(Received for publication, August 21, 1917.)

The temperature coefficients of life processes have within the last few years attracted a good deal of attention.¹ Interest has chiefly centered about the question whether life processes have the temperature coefficients of ordinary chemical reactions and whether investigations of this sort enable us to distinguish between chemical and physical processes (on the ground that in general the latter possess lower temperature coefficients than the former).

In these discussions it is universally assumed that we are dealing with simple chemical reactions. A little consideration shows that this cannot always (or even commonly) be the case. Most substances formed in the organism are also broken down and the amount present must depend on the relative rates of formation and of decomposition. Change of temperature may affect consecutive reactions in an entirely different manner from simple reactions (in which the substance formed is not at once broken down). This may be made clear by a concrete illustration.

Let us suppose that we are measuring the rate at which a substance M decreases as the result of the consecutive reactions $A \rightarrow M \rightarrow B$. Assuming that we have to start with a concentration of $A = 8.853$ and $M = 0.2951$ and that the velocity constant $A \rightarrow B$ is $K_1 = 0.018$ and that of $B \rightarrow C$ is $K_2 = 0.540$, we can calculate the amount of M , as explained in a previous paper, by the formula.²

$$M = 0.2951 (e^{-K_2 T}) + 8.853 \left(\frac{K_1}{K_2 - K_1} \right) (e^{-K_1 T} - e^{-K_2 T})$$

¹ Kanitz, A., *Temperatur und Lebensvorgänge*, Berlin, 1915.

² Osterhout, W. J. V., *Proc. Am. Phil. Soc.*, 1916, lv, 533.

in which T is time and e the basis of natural logarithms. The values obtained² are given in Table I (Curve I).

Let us now consider the effect of raising the temperature 10°C. If both reactions have the temperature coefficient 2, K_1 becomes 0.036 and K_2 becomes 1.080. The values of M under these conditions are given in Table I (Curve II). Inspection of the tables, and of the curves in Fig. 1, shows that at the higher temperature it requires just half as long to produce the same amount of chemical action as at the lower. Hence the consecutive reaction appears to behave in this instance like a simple reaction.

TABLE I.

Time.	Value of M .			
	Curve I. $K_1 = 0.018$ $K_2 = 0.540$	Curve II. $K_1 = 0.036$ $K_2 = 1.080$	Curve III. $K_1 = 0.0216$ $K_2 = 1.080$	Curve IV. $K_1 = 0.036$ $K_2 = 0.648$
min.				
10	87.76	74.96	64.39	120.70
20	74.96	55.32	53.82	87.31
30	64.26	41.62	45.31	63.94
40	55.32	32.06	38.45	47.63
50	47.86	25.35	32.92	36.26
60	41.62	20.74	28.47	28.32
90	28.43	13.65	19.62	16.22

The result will be quite different if the two reactions have different temperature coefficients. Let us suppose that the speed of the reaction $A \rightarrow M$ is determined by diffusion (as happens in some heterogeneous reactions) and has in consequence a low temperature coefficient which we will assume to be 1.2. Assuming that the reaction $M \rightarrow B$ has a temperature coefficient 2 we find that on raising the temperature 10°C. K_1 becomes 0.0216 and K_2 becomes 1.080. The values of M under these conditions are given in Table I (Curve III).

Comparison of the values and the curves (Fig. 1) shows that the times required for equal amounts of chemical action have no constant relation in Curves I and III. At one point on Curve III less than half as much time is required for a given amount

² For convenience in previous calculations the results were multiplied by 305 and 10 was added. This makes the value of M at the start equal 100.

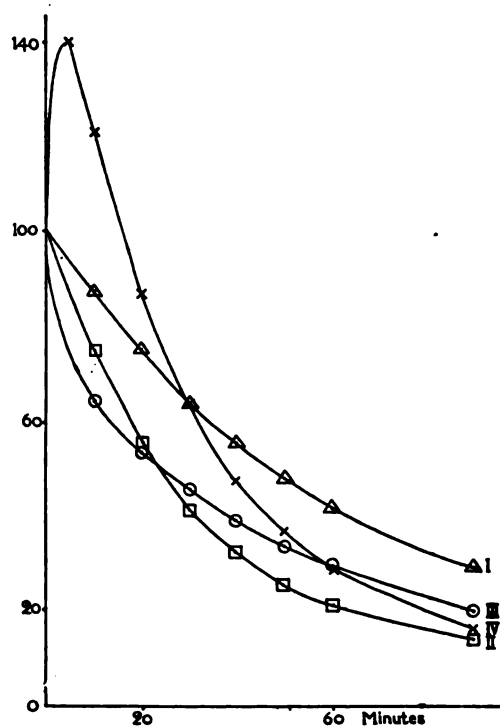


FIG. 1. Curves showing the rate of disappearance of a substance when the reactions by which it is formed and decomposed have the velocity constants given below.

Curve.	Rate of formation = K_1 .	Rate of decomposition = K_2 .
I	0.018	0.540
II	0.036	1.080
III	0.0216	1.080
IV	0.036	0.648

Curves II, III, and IV are derived from Curve I by assuming that the temperature is raised $10^\circ\text{C}.$; if the two reactions have the temperature coefficient 2 we obtain Curve II; if the coefficients are 1.2 and 2 respectively we obtain Curve III; if the coefficients are 2 and 1.2 respectively we obtain Curve IV.

of chemical action as on Curve I; at another point one-half as much is required and at another point approximately three-fourths. It is also noticeable that the form of Curve III is quite different from that of Curve II, as shown by the fact that the curves cross.

Let us now consider the effect when the temperature coefficient of the first reaction is 2 and that of the second is 1.2. On raising the temperature 10°C. K_1 becomes 0.036 and K_2 becomes 0.648. The values of M are given in Table I (Curve IV). The form of the curve is quite different from that of the others in that there is first a rise followed by a fall. In experimental work the short period of rise might be overlooked or regarded as due to experimental error or some disturbing ("inhibiting") factor, such as is commonly assumed to account for delay at the beginning of a reaction.

If the observer supposed that he had to do with a simple reaction of the type $M \rightarrow B$ and proceeded to calculate the velocity constant he would obtain the values given in Table II.⁴

TABLE II.
Apparent Velocity Constants Obtained on the Supposition That the Process Is a Simple Reaction.

Time.	Apparent velocity constant.			
	Curve I. $K_1 = 0.018$ $K_2 = 0.540$	Curve II. $K_1 = 0.036$ $K_2 = 1.080$	Curve III. $K_1 = 0.0216$ $K_2 = 1.080$	Curve IV. $K_1 = 0.036$ $K_2 = 0.648$
min.				
10	0.0064	0.013	0.022	
20	0.0071	0.014	0.016	0.0033
30	0.0073	0.015	0.014	0.0074
40	0.0075	0.015	0.013	0.0095
50	0.0075	0.015	0.012	0.011
60	0.0076	0.015	0.012	0.012
90	0.0077	0.015	0.011	0.013

⁴ The calculations were made by the use of the formula

$$K = \frac{1}{T} \log \left(\frac{a}{a-x} \right)$$

employing common logarithms. We put $A = (100 - 10) = 90$ and $a - x = M - 10$. The subtraction of 10 is necessary because 10 was previously added. (See foot-note 3.)

A consideration of these values is very instructive. It is evident that when the relation $K_2 \div K_1$ has a certain value (as in Curves I and II where $K_2 \div K_1 = 30$) the reaction appears to proceed as a monomolecular reaction which is somewhat "inhibited" at the start,⁵ while with other values it may appear to be greatly inhibited at the start (Curve IV, $K_2 \div K_1 = 18$) or to go much faster in the beginning than is expected (Curve III, $K_2 \div K_1 = 50$).

These facts deserve consideration in interpreting the temperature coefficients of consecutive reactions, to which category many life processes undoubtedly belong.

SUMMARY.

Where substances are formed and at once broken down, as happens in many life processes, interesting conditions may arise if the reaction which forms the substance has a different temperature coefficient from that which destroys it.

⁵ Mellor, J. W., Chemical Statics and Dynamics, London, 1909, 113.

A BIOLOGICAL ANALYSIS OF PELLAGRA-PRODUCING DIETS.

I. THE DIETARY PROPERTIES OF MIXTURES OF MAIZE KERNEL AND BEAN.*

BY E. V. MCCOLLUM AND N. SIMMONDS.

(From the Laboratory of Agricultural Chemistry of the University of Wisconsin, Madison.)

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INTRODUCTION.

The time has now arrived when the third period of our studies of nutrition may be appropriately introduced. The first phase of the work dealt with an extensive inquiry into the cause of nutritive failure in animals restricted to diets of purified proteins, carbohydrates, fats, and inorganic salts. After careful consideration of the suggestive work of Hopkins,¹ Stepp,² and Funk,³ we arrived at the conclusion that there were lacking in such mixtures two substances or groups of substances, the chemical natures of which are still unknown, which the diet of an animal must contain if its life is to be maintained. One of these (fat-soluble A) is soluble in fats and is found in most concentrated form in butter fat and egg yolk fats. Its distribution in other foods is mentioned below. The second substance is never associated with fats in foods, but is everywhere present in natural foods in relative abundance. It is readily supplied in experimental rations by the addition of alcoholic extracts of natural foods, but it is also soluble in water. This we call water-soluble B.

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¹ Hopkins, F. G., *J. Physiol.*, 1912, xliv, 425.

² Stepp, W., *Biochem. Z.*, 1909, xxii, 452; *Z. Biol.*, 1912, lvii, 135; 1913, lix, 366.

³ Funk, C., and Cooper, E. A., *Lancet*, 1911, ii, 1266.

Phosphorized proteins have been shown to have no superior value over others which contain no phosphorus, and a long list of substances including complex lipoids, pigments, sulfatides of the nervous tissues, components of the several prosthetic groups with which body proteins are combined are now all known to be produced within the tissues and do not need to be supplied in the food. The synthetic capacity of the animal cell is thus shown to be immeasurably greater than was formerly supposed, but all the evidence still supports the view that nearly all of the amino-acids formed on digestion of proteins are indispensable components of the diet. Furthermore, the animal tissues are very dependent upon certain of the finer stereochemical structures in both the amino-acid and in the carbohydrate groups which serve them as food complexes.

By finding just how far the diet can be simplified and still be biologically complete we have made it clear that, assuming as we do provisionally that the fat-soluble A and water-soluble B are individual substances rather than complex mixtures of a number of physiologically indispensable compounds, the adequate diet for an animal is in reality sufficiently simple to be comprehended and visualized with regard to all its parts.^{4, 14, 15}

Our second undertaking was to examine one by one several typical representatives of the classes of natural food substances as the seeds, leaves, fruits, and tubers of plants. We have shown for a sufficient number of these the exact nature of the additions of simple and unidentified factors A and B which must be made in order to render one of these single food substances complete from the nutritive standpoint. Some of these studies are still in progress but we have progressed so far that we feel confident that all the important generalizations have been made regarding the special properties of our natural foodstuffs and the lines along which we must proceed in order to make up satisfactory diets.^{7, 16}

It is already clear from these studies that serious mistakes are being made in the choice of foods, especially by the poor in many parts of the world. Beri-beri, scurvy, rickets, and pellagra have all been recognized as being referable to faulty diet although

⁴ McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xxiii, 231.
McCollum, E. V., and Kennedy, C., *ibid.*, 1916, xxiv, 491.

different opinions still prevail regarding the etiology of the latter disease. Eykmann's experimental production of beri-beri and its cure by dietetic measures,⁵ and the subsequent extensive study by Funk and others of the nature of the curative substance, led to the formulation of the "vitamine" theory by Funk.³ He attributes each of the above mentioned syndromes to a lack of a specific chemical substance (vitamine) and this theory is so attractive that it has gained widespread acceptance. Our studies of the nature of the dietary deficiencies of many natural food-stuffs forced upon us the conclusion that there are under actually existing conditions three other dietary factors which are responsible for malnutrition in both man and animals.⁶ Indeed the conclusion is necessary that except in beri-beri among those who eat polished rice almost exclusively, and in the very rare disease xerophthalmia which we attribute to specific starvation for the fat-soluble A the "vitamine" hypothesis finds no application. In a recent paper McCollum and Pitz have considered the etiology of experimental scurvy from an entirely different viewpoint.⁶

In the present series of papers we shall describe a very elaborate experimental inquiry concerning the properties, with respect to the several dietary factors, of diets of the complexity usual in human nutrition and derived from all the more important food materials (except milk and eggs) in common use in the United States. These, and milk in particular, form a wonderful safeguard in human nutrition and a most valuable adjuvant to all rations employed in animal production. The specific reasons for this will be apparent as the subject is developed. It will be brought to light how, when milk is absent from such diets as are not infrequently employed, the quality of food mixtures affording a fairly wide variety is frequently so poor as to bring us near the line where visible signs of malnutrition become manifest. A biological analysis of such diets will render possible their correction by relatively simple means.

⁵ Eykmann, C., *Arch. Hyg.*, 1906, lviii, 150; *Arch. Path. Anat.*, 1897, cxlviii, 523.

⁶ McCollum, E. V., and Pitz, W., *J. Biol. Chem.*, 1917, xxxi, 229. In the second paper of this series we discuss reasons why xerophthalmia is to be looked upon as the second and aside from beri-beri the only "deficiency" disease in the sense of Funk.

In a recent paper we have discussed the points of resemblance in the dietary properties of seeds in general as contrasted with the leaf of the plant, and have pointed out the lines along which it is necessary to proceed in order to make up from vegetable sources diets which will promote growth and physiological well-being.⁷ The most important generalizations which can be made concerning the seeds as a group are the following.

1. No seed which we have studied, when fed as the sole source of nutriment, can induce growth or prolonged well-being in a young animal. Mixtures of seeds are more satisfactory in maintaining animals in a fair state of health without growth than are seeds of a single species of plant as the sole food, but even complex mixtures of seeds fed with distilled water (salt-free) will not support growth in young rats.⁷

2. The inorganic content of each of the seeds we have studied (wheat,⁸ oat,⁹ maize,¹⁰ rice,¹¹ wheat germ,¹² bean,¹³ pea,¹⁴ flax seed,¹⁵ and millet seed¹⁶) is so constituted with respect to total quantity and quantitative relationships among its constituents that certain salt additions are essential before growth can proceed in young animals. This is true for mixtures of seeds as well as for the seeds of a single variety.⁷

3. The seeds of the wheat, oat, maize, rice, bean, and pea are too low in their content of a dietary essential, the nature of which is still unknown, to supply the needs of a young animal

⁷ McCollum, E. V., Simmonds, N., and Pitz, W., *J. Biol. Chem.*, 1917, xxx, 13.

⁸ Hart, E. B., McCollum, E. V., Steenbock, H., and Humphrey, G. C., *Wisconsin Agric. Exp. Station Research Bull.* 17, 1911. Hart and McCollum, *J. Biol. Chem.*, 1914, xix, 373. Hart, Miller, and McCollum, *ibid.*, 1916, xxv, 239. McCollum, Simmonds, and Pitz, *ibid.*, 1916-17, xxviii, 211.

⁹ McCollum, Simmonds, and Pitz, *J. Biol. Chem.*, 1917, xxix, 341.

¹⁰ Hart and McCollum, *J. Biol. Chem.*, 1914, xix, 373. McCollum, Simmonds, and Pitz, *ibid.*, 1916-17, xxviii, 153.

¹¹ McCollum and Davis, *J. Biol. Chem.*, 1915, xxiii, 181.

¹² McCollum, Simmonds, and Pitz, *J. Biol. Chem.*, 1916, xxv, 105.

¹³ McCollum, Simmonds, and Pitz, *J. Biol. Chem.*, 1917, xxix, 521.

¹⁴ McCollum, Simmonds, and Pitz, unpublished data.

¹⁵ McCollum, Harvey Society Lecture, *J. Am. Med. Assn.*, 1917, lxviii, 1379-1386.

¹⁶ McCollum, Simmonds, and Pitz, *Am. J. Physiol.*, 1916, xli, 333, 361.

during growth. This substance is present in abundance in certain fats as butter fat, egg yolk fats, and the fats extracted by solvents from animal organs such as the liver and kidney, free from visible fats. It likewise occurs in abundance in the leaves of plants,¹⁶ and in considerable amounts in the *seeds* of the flax and millet plants.¹⁶ This dietary essential we have provisionally termed "fat-soluble A."⁴ It is not extracted from plants with the fats by such solvents as ether, chloroform, benzene, or acetone, and is therefore not found in any fats or oils of plant origin.¹⁶ Hot alcohol does remove it from plant tissues.¹⁰

4. All seeds which we have studied contain protein mixtures which are of relatively poor quality when the proteins of a single kind of seed serve as the sole source of proteins. Mixtures of seeds in certain cases yield protein combinations which mutually make good each other's amino-acid deficiencies and raise their biological value.⁷

5. Each of the seeds which we have studied can be supplemented by highly purified protein, pure inorganic salts, and one of the growth-promoting fats, so as to form a satisfactory ration for growth and maintenance. 15 to 25 per cent of any one of the seeds which we have enumerated, will, when fed with a mixture of purified food substances, serve to supply all unidentified dietary factors which are soluble in water or in alcohol (we have evidence that there is but one such indispensable substance). This we have provisionally designated as "water-soluble B."⁴ This one is never associated with fats of either animal or plant origin.

6. The leaf of the plant, if we may generalize from studies of three types, alfalfa, clover, and cabbage, differs from the seed in having a high content of inorganic elements and is particularly rich in those elements, calcium, sodium, and chlorine, which are found in but small amounts in the seed. The leaf is much richer in the fat-soluble A than are such seeds as wheat, oat, maize, bean, and pea. Leaf and seed supplement each other with respect to these two dietary factors. Since in many instances at least the leaf and seed mixtures have been shown to yield adequate protein mixtures there is probably always some supplementary relationship between them with respect to the amounts of the various amino-acids which they yield on digestion.^{7, 16}

In our studies already reported no systematic data have been presented which show the dietary properties of mixtures of cereal and legume seeds. In the present paper we describe the nature of the purified food additions which must be made to combinations of the maize kernel and the white (navy) bean in various proportions, in order to make these seed mixtures dietetically complete. The experiments described in this paper were so planned as to make clear the value of each dietary factor in the maize-bean mixtures as compared with several of the most important seeds especially maize and wheat, and navy bean, which have been most thoroughly studied separately in a manner which showed the nature of their dietary deficiencies.

The charts presented furnish data which may be briefly summarized as follows.

1. Like each of the two seeds individually,^{10, 13} their mixtures contain too small an amount of fat-soluble A to induce optimum well-being in growing animals.

2. The mixtures of maize and beans furnish a great abundance of the second unidentified dietary factor water-soluble B.

3. The most satisfactory protein mixture attainable with mixtures of these two seeds is found in about 80 per cent of maize and 20 per cent of beans (Chart 3, Lot 745; Chart 17, Lot 814). This protein mixture has little more value biologically than an equivalent amount of maize kernel proteins, for rats are able to grow at about half normal rate on a diet which contains but 9 per cent of protein all derived from the maize kernel. At lower levels (*e.g.*, 7 per cent of the food mixture) the proteins of the wheat and the maize kernel are of about the same value. When a ration is properly constituted with respect to other factors and contains 9 per cent of wheat proteins growth may be practically normal over a period of 5 months, after which an injurious effect is apparent in stunting and lack of fertility.¹⁷ It is highly probable that if the maize protein content were raised to 12 per cent approximately normal growth would result. In Charts 5, 7, and 9 the records show that with 12 per cent of a mixture of maize and bean proteins, in which 63 per cent comes from maize and 37 per cent from beans, growth approximates the normal rate, but better

¹⁷ McCollum, Simmonds, and Pitz, *J. Biol. Chem.*, 1916-17, xxviii, 211.

results are secured with the same food mixtures supplemented with pure casein (Charts 6 and 10). This shows that the maize-bean mixture furnishes proteins which require improvement to induce the optimum well-being.

We have shown in a previous publication that 6 per cent of milk proteins in a food mixture just suffice to support growth at the normal rate in rats.¹⁸ It is evident therefore that the protein mixture from maize 80 and beans 20 per cent has just about one-half the biological value for growth that the total protein mixture in milk possesses.

4. A detailed study has been made of the exact nature of the mineral deficiencies of the maize and bean mixtures. The results support in every way the conclusions which we expressed in a recent publication,⁷ in which the supplementary dietary relationships of the leaf and seed were discussed. It was pointed out that sodium and calcium were the elements concerned in determining the unsatisfactory character of the mineral content of seeds in general. This is an extremely important matter in human nutrition and agricultural practice in certain parts of the country where the water used for drinking purposes is nearly free from lime and sodium chloride. Efficient utilization of food by growing animals is not possible when the diet is deficient in these elements.

There are two practices which make for safety in human nutrition in those districts where the water is low in sodium and calcium. These are, first, the consumption of milk as a regular article of diet, and second, the use of the leaves of plants as human food. Milk is always high in calcium and as a rule contains a far greater amount of sodium chloride than do any of the seeds. The same is true of the leaves of plants. It is highly probable that meats do not make good the mineral deficiencies of a diet derived principally from seeds. We cannot emphasize too strongly the importance of using milk as a regular constituent of the human diet and also the great benefit which would result from a more extensive use of the leaves of plants as a staple article of the diet.

The liberal use of pot-herbs by those peoples who approximate

¹⁸ McCollum and Davis. *J. Biol. Chem.*, 1915, xx, 415.

most closely strict vegetarian habits is a practice which saves them from extinction. The cause is now clear in that both the mineral deficiencies and the shortage of the fat-soluble A in the seeds are thus made good.

Investigations by the special methods which we have developed have now reached a point which makes it clear how near the danger line from faulty diet are certain classes of people even in the United States at the present time. After an extensive study of the dietary habits of the people in the South who suffer from pellagra, Goldberger¹⁹ selected for an experiment on human beings a diet of wide variety with a view to the experimental production of pellagra. He considered the diet to represent the type of food consumed by pellagrins before and during the onset of the disease. "The ingredients of this diet were wheat flour (patent), corn meal, corn grits, corn starch, white polished rice, standard granulated sugar, cane syrup, sweet potatoes, pork fat (fried out of salt pork), cabbage, collards, turnip greens, and coffee." Calculated on a wet basis the sweet potatoes, cabbage, and collards made one-fifth of the diet, but on a dry basis only about 4 per cent of the total solids. This amount of leaf is not sufficient to make good the very serious shortage of calcium and sodium in the remainder of the diet nearly all of which was derived from the endosperm of seeds. The common practice of salting human foods doubtless supplied sodium chloride in sufficient amounts. It is evident likewise from our studies of wheat, maize, and rice that the fat-soluble A is not supplied in Goldberger's diet in anything like the amount necessary to support an animal in health. 4 per cent of the solids of the ration as leaves would not furnish enough of this factor for the rat. When it is further considered that the protein content of the diet was not above 8 per cent of the food mixture, there is abundant reason for the enfeeblement and the development of the syndrome of pellagra.

In Chart 7, Lot 792, are shown the records of a group of rats whose diet furnished 12.5 per cent of protein, about 63 per cent of which was derived from the maize kernel and 37 per cent from the bean. All the deficiencies of the mixture from the dietary

¹⁹ Goldberger, J., *J. Am. Med. Assn.*, 1916, lxvi, 471.

standpoint were made good except the protein factor. On this diet the animals looked very old and rough-haired at the age of 8 months. The one female which produced young was incapable of rearing them. After three of the six young in the third litter had died, pure casein was added to her diet. The immediate recovery of the remaining young and their rapid growth proved conclusively that the protein factor was solely responsible for the poor nutrition of the entire group. This diet, which contained only maize and bean proteins (Lot 792), furnished 90 gm. of protein for a 3,000 calorie portion or about a third more than the Chittenden Standard. In the light of such experimental evidence it is entirely unnecessary to invoke a hypothetical "vitamine," the absence of which causes pellagra. We desire to emphasize again that the nature of the deficiencies of the more important seeds and mixtures of the same from the dietary standpoint is now well understood, and that there is grave danger in deriving nearly the whole food supply from the seeds of plants.

Credit is due to Mr. Walter Pitz for assistance in preparing some of the materials employed in these experiments.

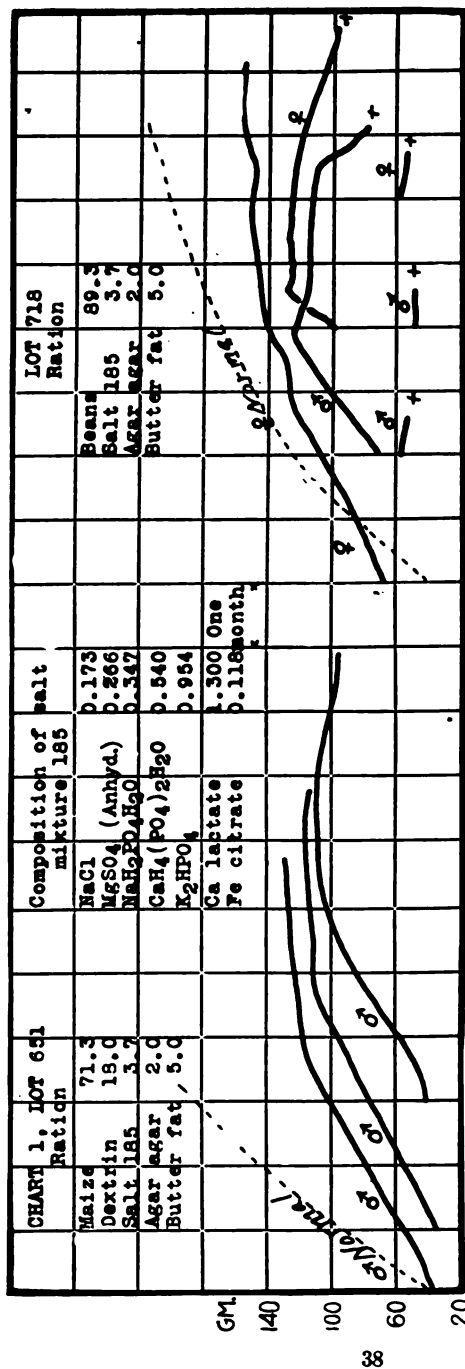


CHART 1. Lot 651 and Lot 718 show the comparative values of the proteins of the maize kernel and of the navy bean respectively. In both rations the deficiencies of the seed except the protein were made good by additions of inorganic salts, and of butter fat (fat-soluble A). The protein content of the maize ration was 7.13 per cent, and of the bean ration 20 per cent. Growth in both cases was very slow and the limiting factor was the amount and character of the protein. In the ration containing 89.3 per cent of bean, there are of course debilitating effects due to fermentation in the intestine.¹³

It is probable that if this depressing factor could be removed, somewhat better growth would be secured with this plane of bean proteins. 7 per cent of maize proteins have approximately the same value as 20 per cent of bean proteins when each seed is fed as the sole source of protein. When mixed the proteins of maize and beans supplement each other's amino-acid deficiencies and mutually enhance each other's biological value. This is shown by Charts 3, 4, 5, 7, 8, 9, 11, 12, 16, 17, 23, and 24.

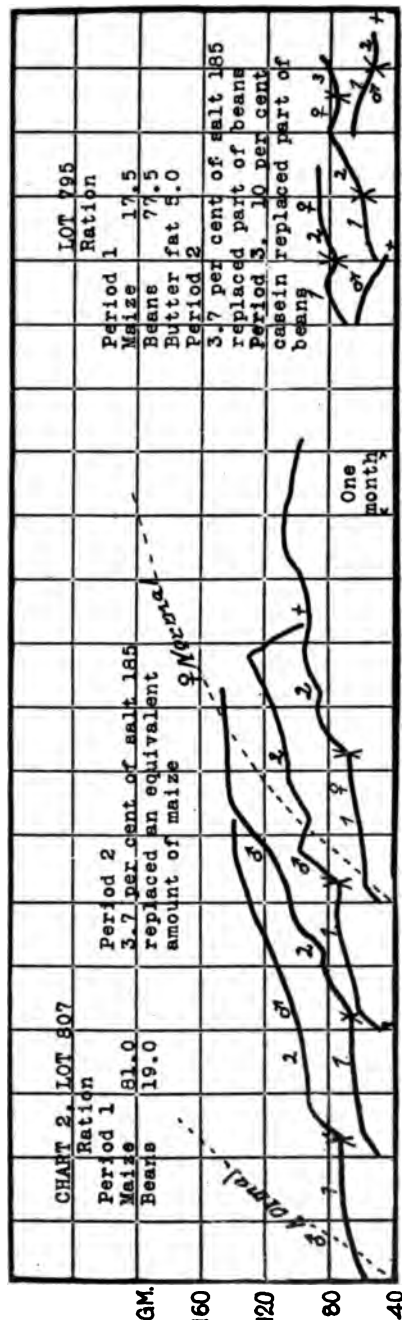


CHART 2. Lot 807. During Period 1 the rats were fed maize 81 per cent and beans 19.0 per cent. No growth was possible. That the inorganic content of these seeds is unsatisfactory for growth is suggested by the noticeable improvement of the animals in Period 2 when 3.7 per cent of a suitable salt mixture replaced an equivalent amount of maize. This is supported by the curves shown in Charts 4, 19, 20, 21, 23, and 25.

Lot 795 illustrates the total failure of rats to grow when fed maize 17.5 per cent and beans 77.5 per cent with butter fat to supply the fat-soluble A. A high bean and low maize mixture is distinctly inferior to the reverse. Even the inclusion of a salt mixture in Period 2 failed to induce growth. The consumption of a diet which consists principally of thoroughly cooked beans is detrimental to health.¹³

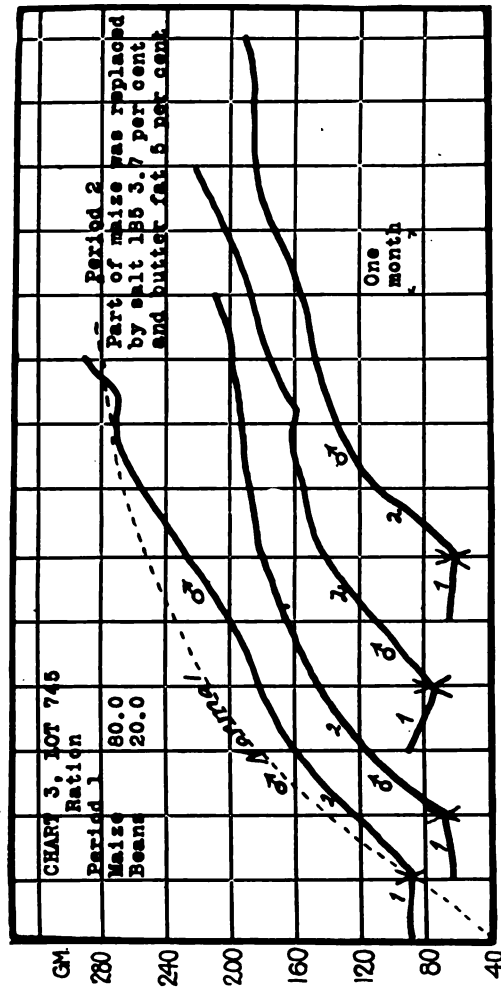


CHART 3. In Charts 9 to 16 inclusive we illustrate the dietary properties of maize and beans in various proportions. For more detailed study we selected the proportions maize 80 and beans 20 per cent. In this mixture 63 per cent of the protein is from maize and 37 per cent from beans.

Lot 745, Period 1, confirms the results with Lot 807 (Chart 2), showing that maize and beans in these proportions cannot support growth when distilled water is supplied. Period 2 illustrates the remarkable improvement which followed when a part of the maize was replaced by a salt mixture and butter fat (fat-soluble A). This ration contained in Period 2 11.73 per cent of protein. Growth was nearly normal. This mixture of protein has a biological value just about half that of milk proteins, for 6 per cent of the latter just suffices to induce normal growth.¹⁸ Charts 4 and 7 show that addition of salts alone or of butter fat alone induces much less growth than do both together.

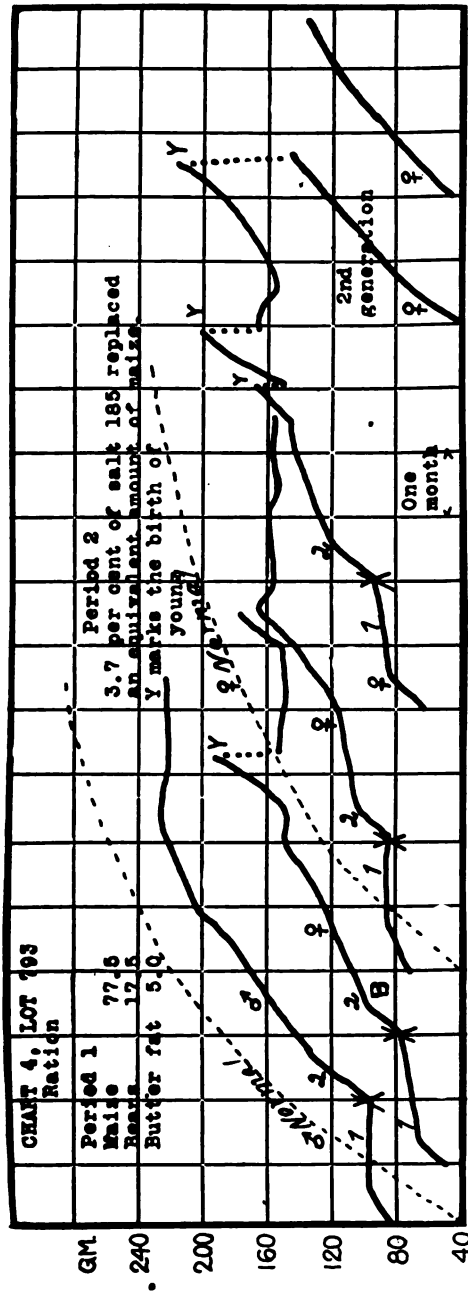


CHART 4. Lot 793 shows that a mixture of maize 77.5, beans 17.5, and butter fat 5 per cent induces almost no growth. After 2 months' suspension of growth the addition of a salt mixture of appropriate composition led to marked acceleration of growth. That the animals were fairly well nourished is shown by the number of litters of young produced, and by the good growth of two of the second generation on this diet. These came from a litter of four. The rat marked B had a litter of nine young. Eight of these weighed collectively 91 gm. at the age of 23 days. 12 per cent of casein was then put into the diet of the mother (Ration 912, Chart 6). The young began to grow rapidly at once and after 33 days more weighed collectively 450 gm. The mother greatly improved in appearance as a result of the improvement of the protein of the diet. It is evident therefore that 11.75 per cent of total protein of which 34 per cent comes from beans and 66 per cent from maize suffices for normal growth but is not good enough to meet the needs of a nursing mother except when the litter is small.

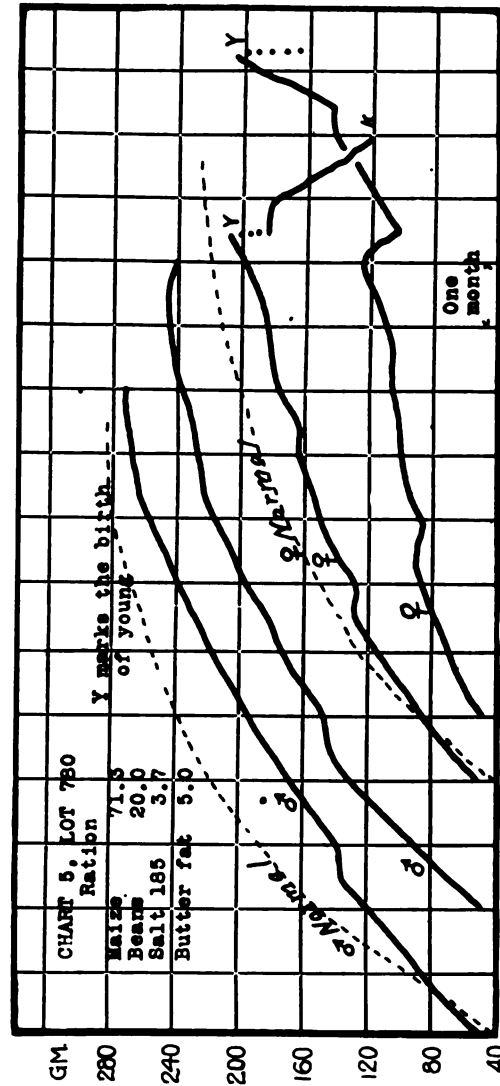


CHART 5. Lot 780 illustrates the character of the growth curves obtained with a diet of maize 71.3, beans 20, salts 3.7, and butter fat 5 per cent. It is evident that the protein mixture is fairly satisfactory, but the fact that there is a tendency for the rats to remain somewhat undersized shows that the protein moiety needs some improvement. That this is true is supported by the results of feeding the maize and bean mixture with salts, fat-soluble A, and protein additions (Chart 6, Lot 912). The two females in Lot 780 each produced but a single litter of young late in life and failed to rear any of them. Further records of the ability to grow and reproduce on 12 per cent of protein, about 65 per cent of which came from maize and 35 per cent from beans, are shown in Chart 7 (Period 2).

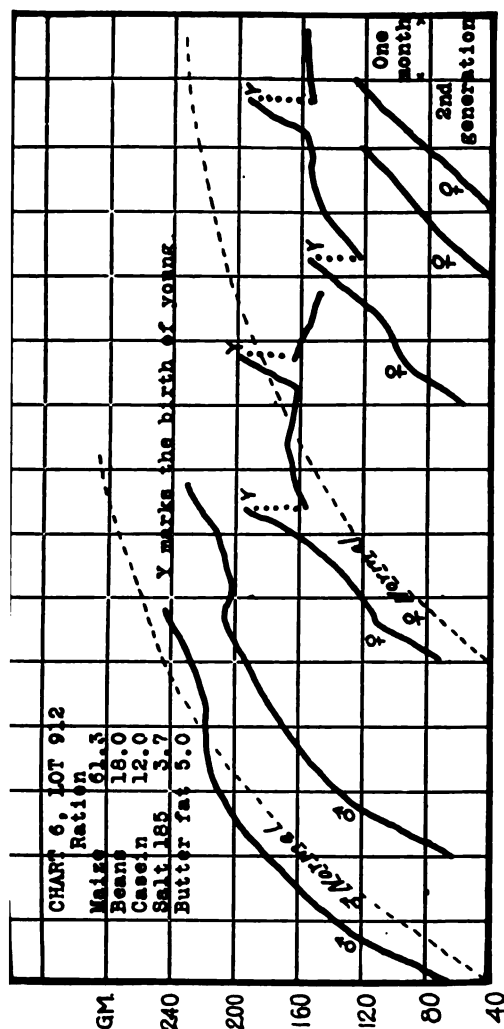


CHART 6. Lot 912 shows the growth curves and reproduction records of rats which received the maize and bean mixture supplemented with three purified food additions; viz., salts, fat-soluble A, and casein. The ration differed from that of Chart 5, Lot 780, in that the protein content was improved by the inclusion of casein. The two females in Chart 5, Lot 780, each produced but a single litter of young at the age of 9 and 11 months respectively, and failed to rear any of them, while each of the two in Lot 912 produced her first litter the 4th month of life and a second litter 2 months later. All four litters were reared (twenty-three young). Two individuals which were continued on the mother's ration grew about normally during 2 months.

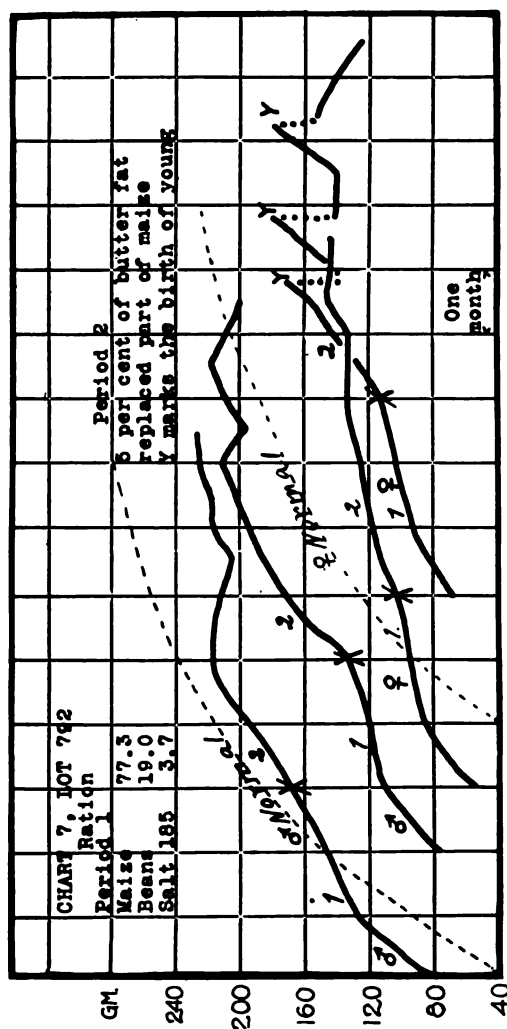


CHART 7. Lot 792 makes it clear that in order to obtain a normal growth curve on our maize 80, bean 20 mixture, it is necessary to add both salts and fat-soluble A. In Period 1 maize, beans, and a salt mixture were fed, and stunting resulted. In Period 2 butter fat was added with the result that marked acceleration of growth took place, followed in the case of one female by the production of three litters of young. The first two litters were dead within a week. The third litter of six weighed but 11 gm. each at the age of 23 days. This is less than half the normal weight for this age. At this point 12 per cent of casein was incorporated in the mother's diet (Ration 912, Chart 6). This led to prompt improvement in three of the young, the others having died. At the age of 57 days these three weighed collectively 253 gm.

After 8 months on this diet which contained 12.5 per cent of its total calories as protein derived from two whole seeds, an amount corresponding to about 90 gm. of protein in a diet furnishing 3,000 calories per day, these rats looked very old, and were drowsy and inactive. Their hair was coarse and rough and stood straight out most of the time.

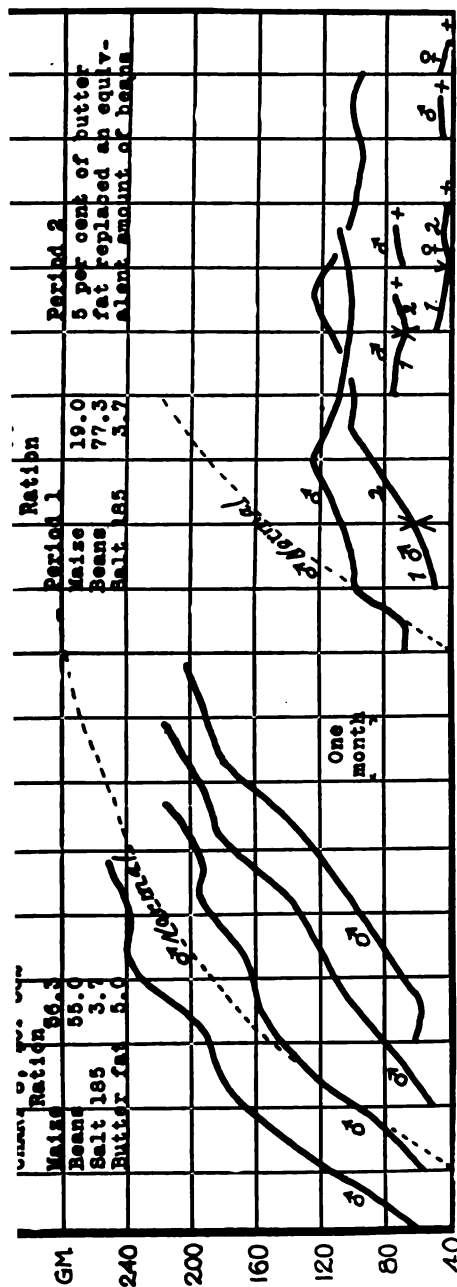


CHART 8. Lot 882 illustrates the fact that when both inorganic salts and fat-soluble A are added, rats can grow at a good rate on a diet containing 55 per cent of beans and 36.3 per cent of maize. This mixture supplies 12.28 per cent of protein of which 22.3 per cent is from the maize kernel and 77.7 per cent from the navy bean. We have shown elsewhere that when properly supplemented 10.35 per cent of bean proteins cannot support appreciable growth. It is evident therefore that an actual supplementary relation exists between the proteins of the maize kernel and the bean. This ration does not promote physiological well-being in a degree near the optimum, notwithstanding the good growth curves of Lot 882. In Chart 14, Lot 749, Period 2, the rats failed to grow well on essentially this food mixture when they had been debilitated by a preliminary period of stunting over 1 month.

Lot 794 shows that when the bean content of the diet is increased to 77.3 per cent and the maize content lowered to 19 per cent (90 per cent of the total protein from bean and 10 per cent from maize) but little growth is possible even when all factors other than protein are properly adjusted. The curve at the left received the diet of Period 2 from the beginning. The interpretation of growth curves obtained with diets high in beans is attended with some uncertainty owing to the injury resulting from excessive fermentation of the hemicelluloses of the bean. We have, however, secured practically normal curves of growth with rations containing 70 per cent of beans, the deficiencies of the proteins of the bean being made good by casein addition.¹² This fact lends weight to the interpretation that this diet (Lot 794) failed to induce growth because of the poor quality of the bean proteins. Maize proteins have only a fairly good supplementary relationship to those of the bean.

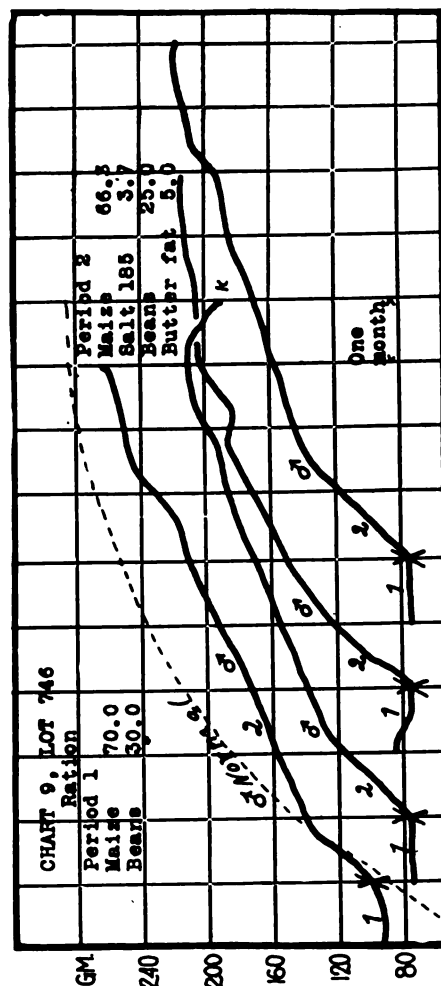


CHART 9. Lot 746 shows the inability of rats to grow when restricted to a diet of maize 70 and beans 30 per cent, and their immediate response with growth when both inorganic salts and butter fat were added. Growth was limited in Period 2 by the poor quality of the protein mixture in the diet. This view is supported by the results of feeding a comparable diet in which 12 per cent of maize was replaced by casein (Lot 913, Chart 10). In the latter case growth was normal and reproduction very successful. (Eight litters, forty-nine young—forty-two were reared.)

This diet (Chart 9) supplied 12.74 per cent of protein of which 33 per cent was from maize and 67 per cent from beans. The records of Lot 746, as well as those of Lots 780, 749, and 706, Charts 5, 14, and 15, all go to show that there is no particular proportion of maize to bean which produces a protein mixture of *high* biological value. The proteins of each seed are, however, somewhat enhanced in value by feeding them together.

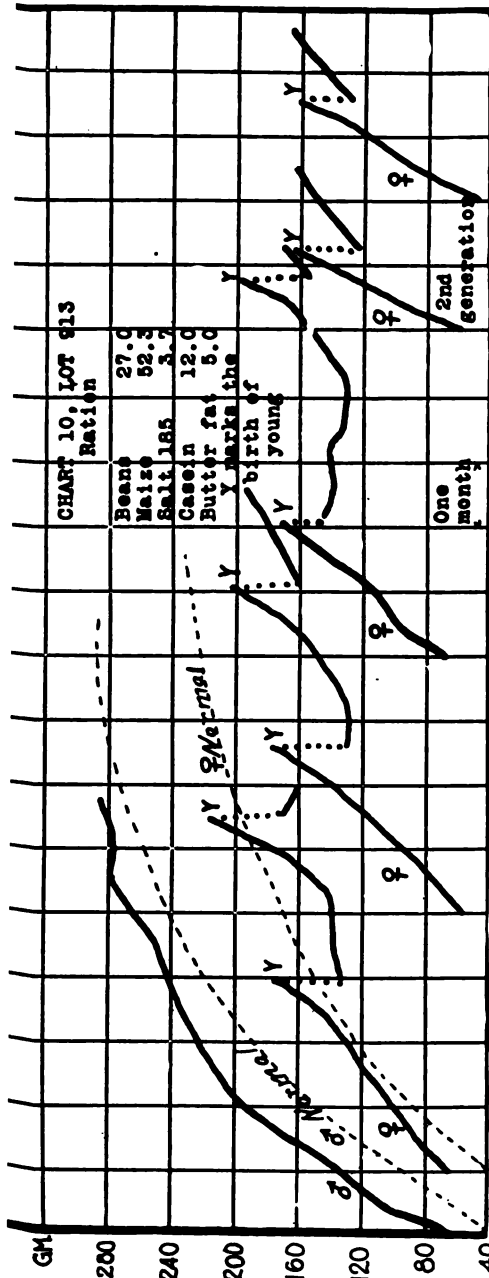


CHART 10. Lot 913 should be considered in comparison with the records of Lot 746 (Chart 9). Maize and bean mixtures show a relatively low protein value as measured by biological tests. This lot showed marked improvement in well-being because of the inclusion of casein, as compared with rats eating a similar diet in which all the proteins came from a mixture of maize and beans.

With mixtures of maize and beans in any proportions, certain inorganic salt additions must be made before any growth can take place when distilled water is given and in order to secure maximum growth fat-soluble A must be added although both seeds appear to contain small amounts of this substance. Successful rearing of the young requires further improvement of the proteins.

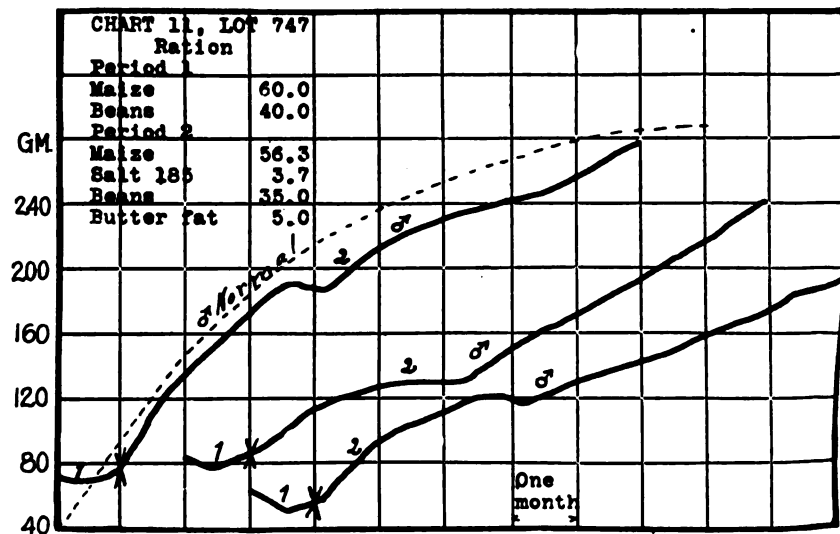


CHART 11. Lot 747, Period 1, shows that maize 60 and beans 40 per cent cannot induce growth in young rats. Mixtures of these two seeds in any proportions require additional fat-soluble A and certain salt additions in order to induce growth. The growth of Lot 747 harmonizes with that of all other experiments we have conducted, in indicating that mixtures of proteins derived from maize and beans in any proportions have a relatively low biological value since when fed with satisfactory supplements, any plane of intake attainable tends to support growth a little below the normal expectation.

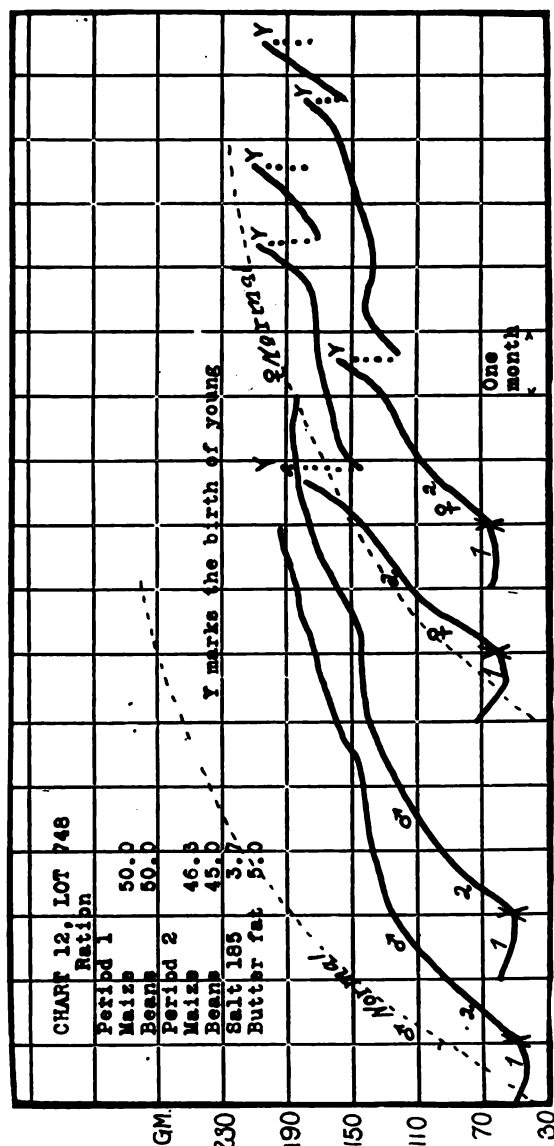


CHART 12. Lot 748 shows total failure of young rats to grow when fed a mixture of maize and beans in equal proportions. Addition of a salt mixture and additional fat-soluble A enabled the animals to grow but the growth was slower in most cases than the normal rate. The long interval between the first and second litters of young indicates nutrition below the optimum. The young were all destroyed the first few days of life. Directly applicable to the support of this interpretation are the superior growth curves of Lots 911 and 883, Chart 13, in which cases the rations were similar but had casein added to supplement the proteins of the maize and beans.

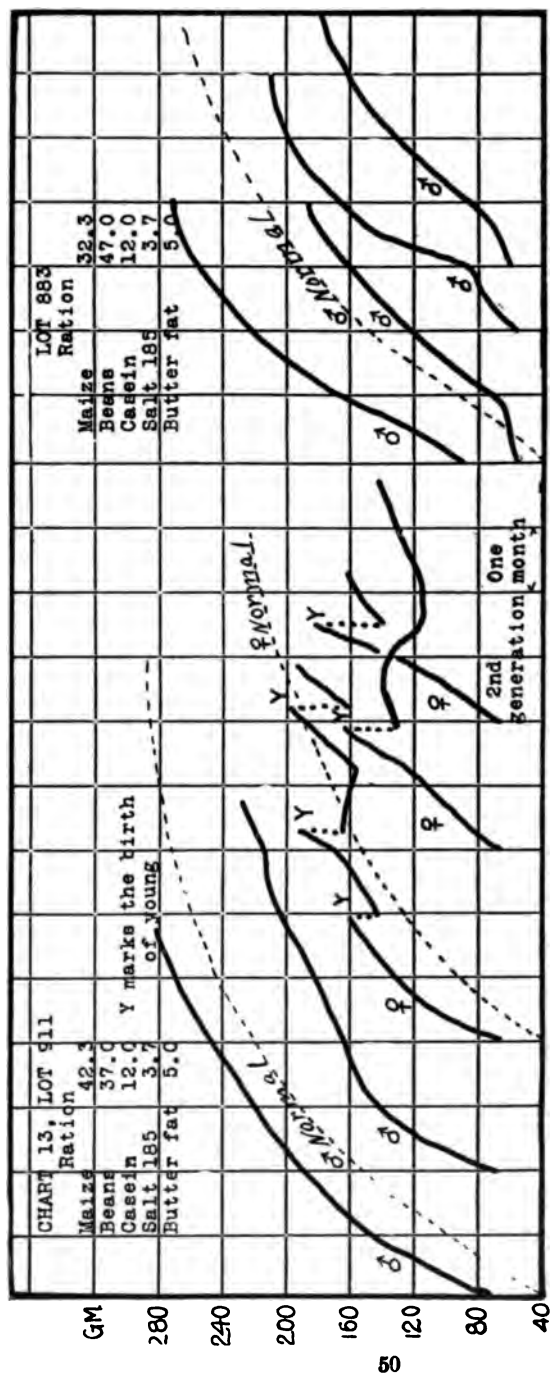


CHART 13. Lot 911 illustrates the good growth and successful reproduction which is attainable with a diet of beans and maize when three dietary factors are improved. Without the salt mixture no growth has been observed on any combinations of maize and beans. Both maize and beans contain too little of the fat-soluble A, and their proteins, no matter in what proportions they are mixed, are of relatively poor quality. These records are comparable with those of Lot 747, Chart 11, Period 2. The latter diet contained no casein and was accordingly inferior.

Lot 883 illustrates the improvement in growth on a mixture of maize 32.3 per cent and beans 47 per cent with casein, salts, and butter fat. The ration is directly comparable with that of Lot 749, Chart 14, Period 3. After a period of stunting, this ration is not good enough to induce a good response with growth, as is shown by the growth in Period 3, Chart 14.

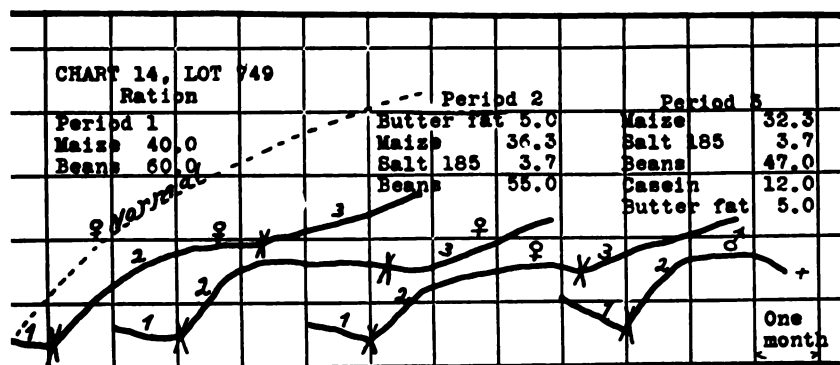


CHART 14. Lot 749. It can be definitely stated that maize and bean mixtures, properly supplemented with respect to the inorganic constituents and fat-soluble A, are better as monotonous rations when maize is the chief constituent than when beans predominate. This appears to indicate a better protein mixture in the former.

A high content of beans tends to cause injury from excessive fermentation so it is not possible to say with certainty that the failure of animals to reverse relations depends principally on the quality of the protein. Chart 15, Lot 796, strongly supports this view.

In Lot 749, Period 3, the inclusion of casein accelerated the rate of growth to a certain extent. Chart 13, Lot 883, shows what animals can do when given this identical mixture from the beginning.

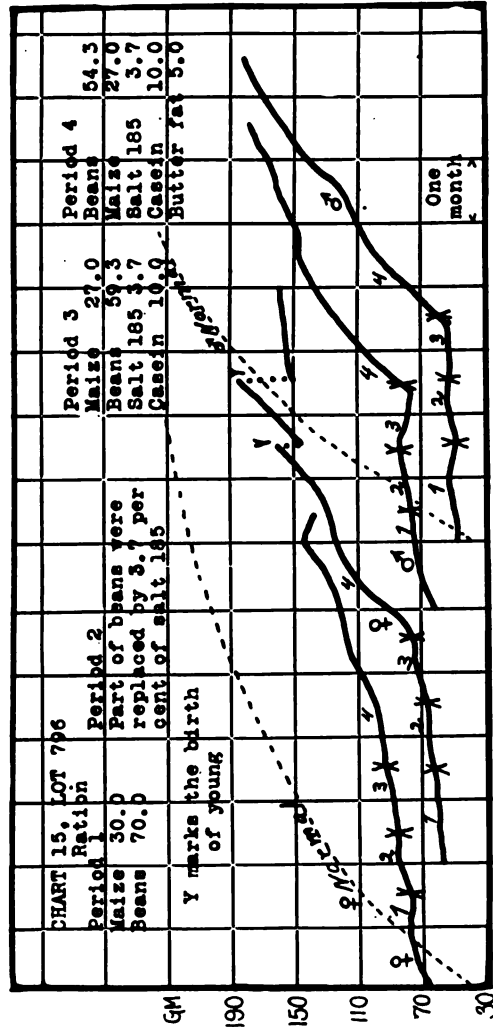


CHART 15. Lot 796. Periods 1, 2, and 3 support the conclusions drawn in the preceding charts; *viz.*, that beans and maize are a total failure as a monotonous ration unless supplemented with respect to at least two dietary factors. Period 3 shows the necessity of adding the fat-soluble A when the content of beans is high, for with beans 59.3, maize 27.0, salts 3.7, and casein 10 per cent, little growth could take place. In Period 4 the inclusion of 5 per cent of butter fat led to growth at a fairly good rate, followed in the case of one female, by the production of two litters of young.

That maize contains some of the fat-soluble A has been shown elsewhere. The bean must likewise contain some of it for Lot 811, Chart 16, has grown to about 80 per cent of the normal adult size and one has reared a few young on a maize and bean mixture supplemented except for the fat-soluble A.

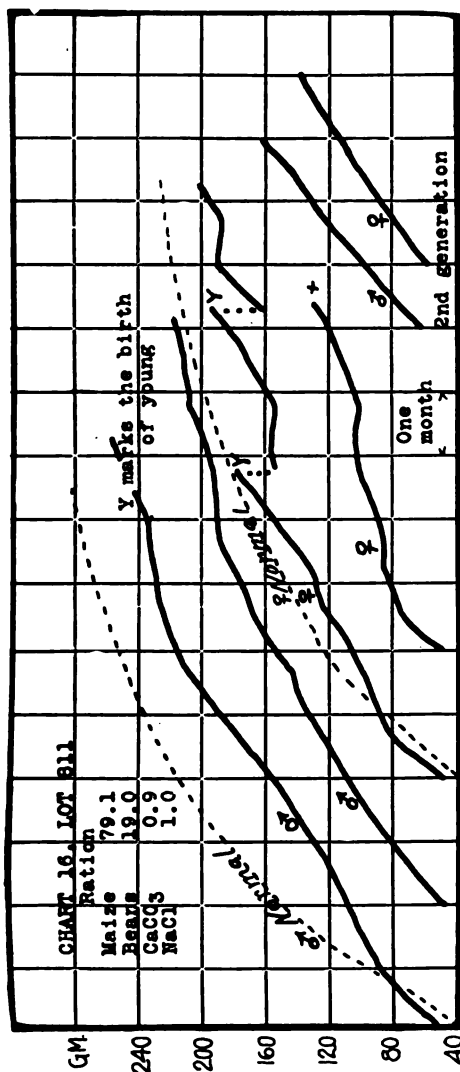


CHART 16. Lot 811 shows that the addition of a complex salt mixture to maize and bean mixtures is unnecessary. The only elements in which such rations as we describe in this paper are lacking are calcium and sodium. This mixture of seeds with no salt additions does not induce growth (Chart 2, Lot 807, Period 1; Charts 3 and 4, Period 1). The failure to grow at the normal rate is here due to a shortage of fat-soluble A. Chart 17, Lot 814, received a similar diet but with 5 per cent of butter fat, and grew at a much faster rate.

It is remarkable that Lot 811 were able to rear four of the twelve young born, although the rats were stunted for lack of the factor A. The young when continued on the mother's diet grew a little too slowly. One female produced no young, which serves to emphasize the fact that the ration was barely able to produce an approximately normal animal. Without a carefully planned series of experiments, relative shortage of a single dietary factor cannot be revealed.

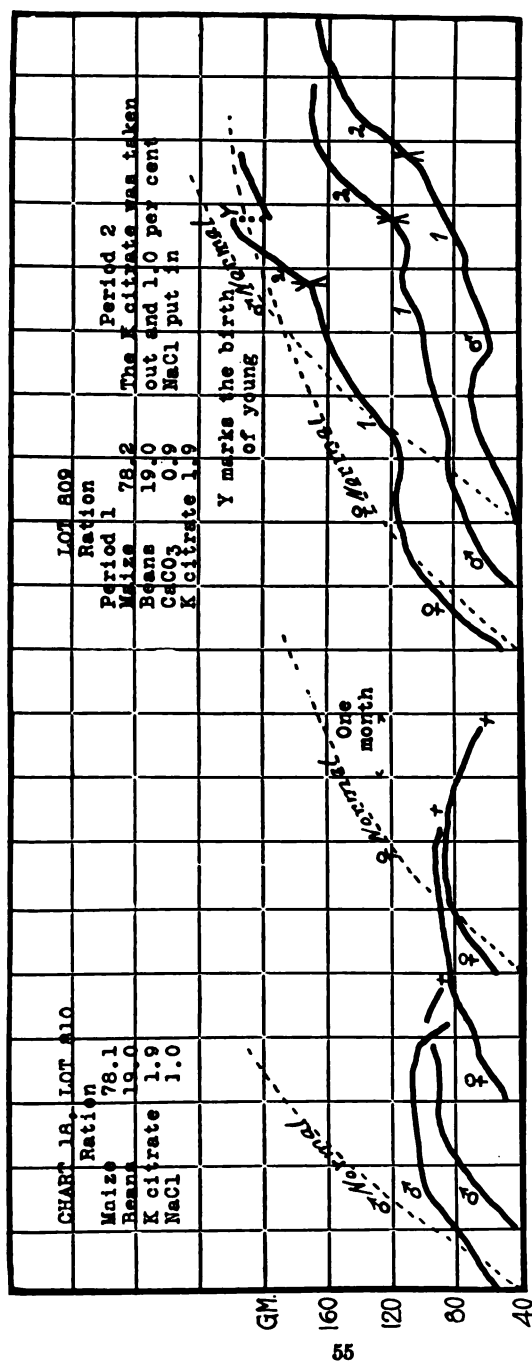
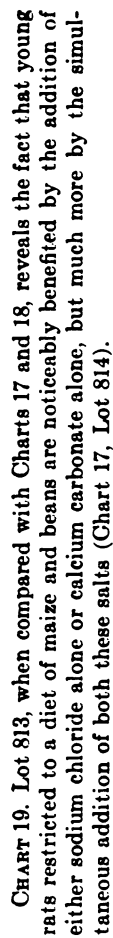


CHART 18. Lot 810 illustrates the almost complete stunting which results from feeding maize and beans to which potassium citrate and sodium chloride were the only inorganic additions made. Marked benefit is observed through the modification of the inorganic content of this ration only when both sodium and calcium are added.

In the case of Lot 809, Period 2, the potassium was taken out and sodium chloride put in. This change caused an acceleration of growth in the stunted animals (see Chart 16, Lot 811).



In Period 2, the addition of calcium increased the rate of growth to some extent but did not enable them to reach normal size, because it appears probable that the animals had been permanently debilitated owing to stunting.

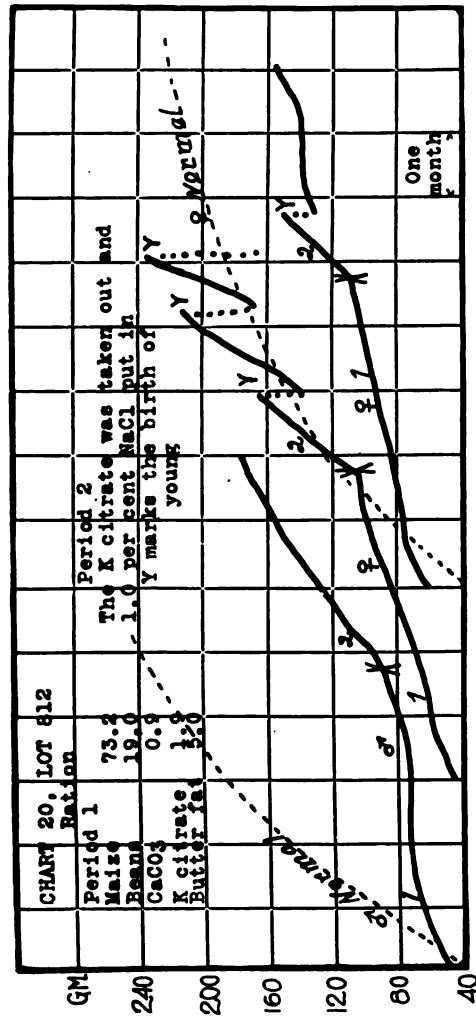


CHART 20. Lot 812 again emphasizes the fact that the maize and bean seeds require both sodium and calcium additions before they can induce growth. The ration of this group had calcium carbonate and potassium citrate as the sole inorganic additions. The ration differed essentially from that of Chart 18, Lot 809, in containing additional fat-soluble A in its butter fat content.

In Period 2 when the potassium was replaced by its atomic equivalent of sodium, there was a rapid acceleration of growth and fertility in the females (Chart 17, Lot 814).

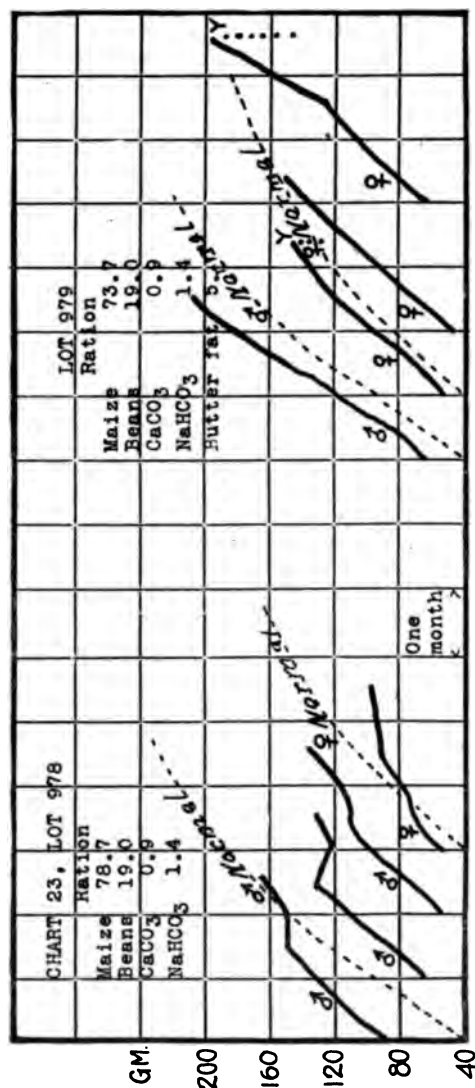


CHART 23. Lots 978 and 979, whose rations were similar except that the latter contained additional fat-soluble A because of the butter fat addition, furnish the final proof that there is in a mixture of maize 80 per cent and beans 20 per cent sufficient chlorine to support growth, when these seeds are fed with sodium and calcium additions and with distilled water so that no accidental inorganic supply is available. As in all other records in this paper, the addition of a little butter fat makes a distinct improvement in the well-being of the animals, which proves that the supply of fat-soluble A in these seeds is below the optimum.

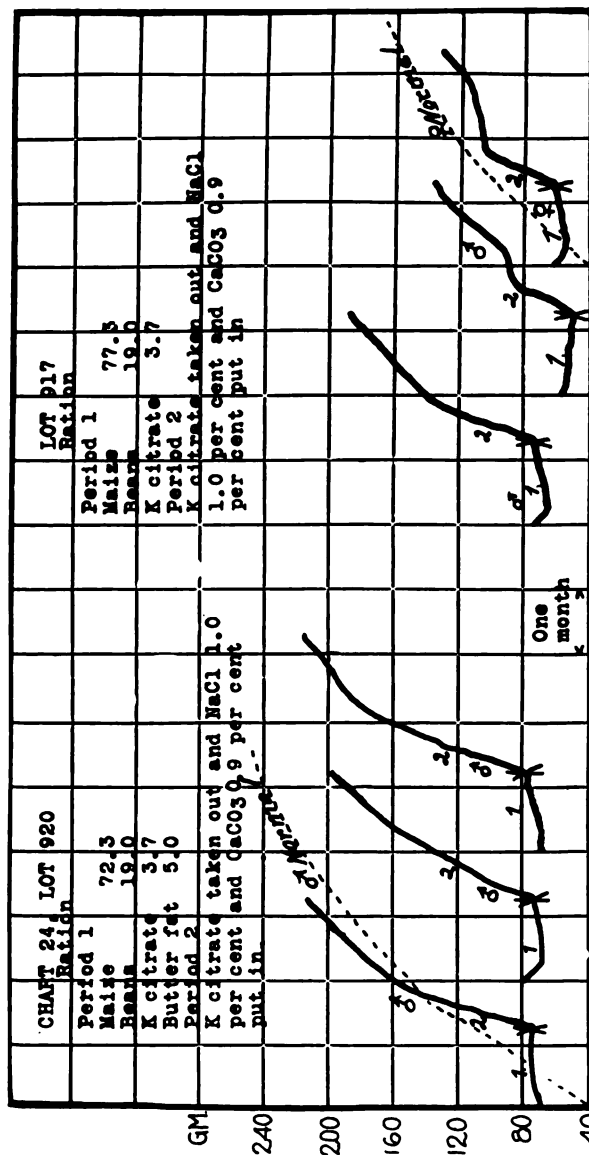


CHART 24. Lots 920 and 917 show in Period 1 the failure of rats to make any growth on a mixture of maize and beans with and without additional fat-soluble A and with potassium as the only inorganic supplement. When in Period 2 the potassium citrate was taken out and both calcium carbonate and sodium chloride put in, growth was at once resumed. The superior condition of the group which had the small butter fat supply over those without it is apparent from the curves.

THE URIC ACID CONTENT OF MATERNAL AND FETAL BLOOD.

By J. MORRIS SLEMONS AND L. JEAN BOGERT.

(From the Departments of Obstetrics and Gynecology and of Pathological Chemistry, Yale Medical School, New Haven.)

(Received for publication, August 14, 1917.)

Determinations of the quantity of uric acid in the blood of mother and fetus at the moment of birth were undertaken as part of a plan to study the placental interchange by comparing the composition of the blood in the two organisms. Thus far, reports have been made concerning the non-protein nitrogen and urea,¹ amino-acids,² sugar,³ and lipoids.⁴ Uric acid determinations were made by the Benedict method, using the procedure recently reported by one of us.⁵

Specimens were secured simultaneously from mother and fetus at the conclusion of the expulsive stage of labor. The maternal blood was aspirated from one of the arm veins; the fetal blood was taken from the umbilical vein and was, therefore, arterial in character.

In normal obstetrical cases practically identical values prevail in the maternal and fetal specimens, a finding in agreement with that of Kingsbury and Sedgwick,⁶ and upon this evidence we believe that uric acid passes through the placenta by the process of diffusion. In this respect, uric acid resembles other nitrogenous waste products, ammonia, urea, creatine, and creati-

¹ Slemons, J. M., and Morriss, W. H., *Bull. Johns Hopkins Hosp.*, 1916, xxvii, 343.

² Morse, A., *Bull. Johns Hopkins Hosp.*, 1917, xxviii, 199.

³ Morriss, *Bull. Johns Hopkins Hosp.*, 1917, xxviii, 140.

⁴ Slemons, J. M., and Curtis, C. S., *Am. J. Obst.*, 1917, lxxv, 569.

⁵ Bogert, L. J., *J. Biol. Chem.*, 1917, xxxi, 165. Acknowledgment is made of suggestions as to technique received from Dr. Louis Baumann of Iowa State University.

⁶ Kingsbury, F. B., and Sedgwick, J. P., *J. Biol. Chem.*, 1917, xxxi, 261.

nine,^{7,8} which are found in equivalent amounts in both circulations. This conclusion also has the support of the findings in pathological cases. Eclamptic mothers in whose blood the quantity of uric acid is twice or three times that of the normal give birth to infants in whose blood the uric acid is correspondingly increased.

Irrespective of parity at the conclusion of labor, the blood uric acid generally falls within normal limits (2 to 5 mg. per 100 cc. of blood). It is noteworthy, however, that higher values are usually found in women giving birth to the first child (primiparæ) than in those who have previously borne children (multiparæ). Thus, the extremes encountered among primiparæ were 1.8 and 8.0 mg., among multiparæ 1.4 and 3.0 mg. A possible explanation of these figures may be the relatively longer and more difficult labor in the case of primiparæ. This possibility is supported by two kinds of evidence: first the fact that we find no difference between the two classes of patients when analyses are made during pregnancy, and second the apparent relationship between the duration and severity of the labor and the quantity of blood uric acid. On the other hand another possible factor in the explanation of this increased uric acid may lie in a temporary renal insufficiency since it is well known that the output of urine is decreased during labor and frequently a mild albuminuria may then be demonstrated.

In view of the possibility that muscular work may increase the uric acid content of the blood, determinations were made before and after labor. Although the number of cases thus far examined is small, the results (Table II) showing an increased uric acid after labor in five out of six cases are interesting and suggestive.

A notable increase in the uric acid of the blood was encountered in a case of syphilis (No. 39), a case of pyelitis (No. 38), and in most cases of toxemia whether of the preeclamptic, eclamptic, or nephritic type. However, one conspicuous exception occurred (Case 36) in which after three convulsions the blood contained 1.9 mg., and after six convulsions 3.4 mg. This woman,

⁷ Hunter, A., and Campbell, W. R., *J. Biol. Chem.*, 1917, xxix, p. xviii.

⁸ Plass, E. D., *Bull. Johns Hopkins Hosp.*, 1917, xxviii, 137.

TABLE I.
Uric Acid Content of the Blood during Pregnancy.

Case.	Para.	Uric acid per 100 cc. of blood.	Period of pregnancy.	Remarks.
		mg.		
1	II	3.8	3rd month.	Neurotic vomiting.
2	II	2.4	5th "	Normal pregnancy.
3	I	2.8	9th "	" " Blood pressure 160.
4	VIII	1.2	Term.	" "
5	VIII	2.3	"	" "
6	II	5.1	"	Syphilis.
7	I	6.0	"	Preeclamptic toxemia. Blood pressure 180.

TABLE II.
Uric Acid Content of Maternal Blood before and after Labor.

Case.	Uric acid per 100 cc. of blood.			Duration of labor.	Remarks.
	First observation.		End of labor.		
		mg.	mg.	hrs.	
3	1 week antepartum.	2.8	6.0	12	Induction of labor.
5	2 weeks "	2.3	3.4	24	" " "
6	Onset of labor.	5.1	6.8	8	Syphilis.
14	" " "	5.9	8.0	36	Normal labor.
30	" " "	6.0	6.0	10	Preeclamptic toxemia.
36	" " "	1.9	3.4	5	Nephritis. Six convulsions. Induction of labor.

TABLE III.
Uric Acid Content of Maternal and Fetal Blood in Normal Primiparous Cases.

Case.	Duration of labor.	Length of second stage.	Source.	Uric acid per 100 cc. of blood.	Remarks.
	<i>hrs.</i>	<i>hrs.</i>		<i>mg.</i>	
8	6	$\frac{1}{2}$	M. F.	1.8	Normal labor; whiffs of chloroform. Normal infant.
9	8	$\frac{1}{2}$	M. F.	4.5	"
10	9	1	M. F.	3.4	"
11	15	1	M. F.	6.8 5.0	"
12	25	1 $\frac{1}{2}$	M. F.	4.8 4.5	Breech presentation; chloroform. Normal infant.
13	25	2	M. F.	4.3 4.3	Normal labor; whiffs of chloroform. Normal infant.
14	36	2	M. F.	8.0 8.1	"
15	24	1	M. F.	5.9 5.6	"

TABLE IV.
Uric Acid Content of Maternal and Fetal Blood in Normal Multiparous Cases.

Case.	Para.	Duration of labor.	Length of second stage.	Source.	Uric acid per 100 cc. of blood.	Remarks.
		hrs.	hrs.		mg.	
16	II	19	1	M.	2.3	Normal labor; no anesthesia.
				F.	2.5	Normal infant.
17	II	12	$\frac{1}{2}$	M.	1.8	Whiffs of chloroform.
				F.	1.8	Normal infant.
18	II	8	$\frac{1}{2}$	M.		"
				F.	2.8	
19	III	6	$\frac{1}{2}$	M.	2.2	No anesthesia.
				F.	2.0	Normal infant.
20	III	4	$\frac{1}{2}$	M.		"
				F.	2.6	
21	III	12	1	M.	3.0	"
				F.	3.3	
22	IV	11	$\frac{1}{2}$	M.	2.0	Whiffs of chloroform.
				F.	2.7	Normal infant.
23	V	10	$\frac{1}{2}$	M.	2.2	No anesthesia.
				F.	2.4	Normal infant.
24	VIII	5	$\frac{1}{2}$	M.	1.4	"
				F.		
25	X	4	$\frac{1}{2}$	M.	2.8	"
				F.	2.2	

TABLE V.
Uric Acid Content of Maternal and Fetal Blood in the Presence of Complications.

Case.	Para.	Source.	Uric acid per 100 cc. of blood.	Remarks.
			mg.	
26	IV	M.	2.6	Forceps; deep chloroform anesthesia.
		F.	2.6	Normal infant.
27	III	M.	2.5	Induction of labor because of size of fetus.
		F.	4.5	Normal infant.
28	VIII	M.	3.4	"
		F.	3.6	
29	I	M.	8.1	Preeclamptic toxemia. Blood pressure 180. Albumin 1.8 per cent.
		F.	6.3	Premature infant.
30	I	M.	6.0	Preeclamptic toxemia. Blood pressure 180. Albumin 1.5 per cent.
		F.	6.1	Normal infant.
31	I	M.	8.1	Preeclamptic toxemia. Blood pressure 190. Albumin 0.9 per cent.
		F.	8.2	Normal infant.
32	I	M.	6.0	Induction of labor. Blood pressure 170.
		F.	5.7	Normal infant.
33	I	M.	8.3	Eclampsia. Three convulsions.
		F.	9.0	Normal infant.
34	I	M.	9.1	Eclampsia. Five convulsions.
		F.		Stillborn, premature infant.
35	III	M.	4.9	Nephritis. Induction of labor.
		F.	7.4	Premature infant.
36	V	M.	3.4	Nephritis. Six convulsions.
		F.	3.0	Premature infant.
37	X	M.	4.5	Nephritis. Spontaneous labor.
		F.	4.8	Normal infant.
38	I	M.	7.4	Pyelitis. At term.
		F.	7.0	Normal infant.
39	II	M.	6.8	Syphilis.
		F.	6.2	Living infant at term.
40	X	M.	6.9	Cardiorenal disease. Spontaneous labor.
		F.		Stillborn infant.
41	III	M.	1.8	Twin pregnancy. Hydramnios.
		F.	3.2	Premature infants, 7th month.
		F.	3.1	

in her fifth pregnancy, was probably suffering from nephritis. In other cases of nephritis high values were found and, therefore, the blood uric acid does not serve to differentiate nephritis from eclampsia—a clinical requirement which obstetricians are constantly seeking to fulfill.

CONCLUSIONS.

The uric acid content of the blood during uncomplicated pregnancy falls within the limits accepted as normal at other times (2 to 5 mg. per 100 cc. of blood).

Larger values were found in cases of syphilis, pyelitis, and the toxemias of pregnancy.

At the conclusion of labor higher values usually prevail in primiparous than in multiparous women. This may be explained by the longer duration and the more energetic character of the labor in the case of the first birth.

In several cases an increased uric acid content of the blood after labor was demonstrated.

Equivalent values for the uric acid in the blood of mother and fetus indicate that uric acid passes through the placenta by diffusion.

As a rule the uric acid of the blood is notably increased in cases of eclampsia and also of nephritis during pregnancy; consequently, these clinical complications may not be distinguished by estimation of the blood uric acid.

THE SCHNEYER METHOD FOR THE DETERMINATION OF LACTIC ACID IN URINE.

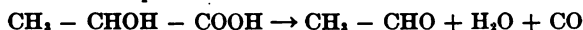
By MARY E. MAVER.

(From the Otho S. A. Sprague Memorial Institute Laboratory of the Children's Memorial Hospital, Chicago.)

(Received for publication, August 2, 1917.)

Meissner¹ in 1915 described a method for the determination of lactic acid in organ extracts, which Schneyer² has applied to the determination of lactic acid in urine. The method, as given by Schneyer, is as follows.

To 250 cc. of urine are added 2 cc. of cold saturated CaCl₂ and 15 to 20 drops of a 10 per cent ammonia solution. The mixture is heated and filtered. If it has been previously ascertained that diacetic acid and acetone are present, the filtrate is boiled for 15 minutes with mineral acid. The filtrate is then evaporated on a water bath to a syrup. The syrup is acidified with 5 cc. of H₃PO₄, about 13 per cent, and mixed with CaSO₄ to a dry crumbling powder. The powder is transferred quantitatively to an extraction thimble and extracted for 8 to 10 hours. The ether is evaporated from the extract and the residue is treated with concentrated H₂SO₄ in a current of carbon dioxide. The carbon monoxide evolved is collected in a nitrometer over 50 per cent KOH. The amount of lactic acid is calculated from the equation:



In our experiments, we followed the suggestion of Meissner, of testing the carbon monoxide for impurities by absorption in ammoniacal cuprous chloride solution.

Schneyer states that: (1) Hippuric and benzoic acids give no carbon monoxide on treatment with concentrated H₂SO₄.

(2) β -Hydroxybutyric and fatty acids develop carbon monoxide in such minute quantities that the error from this source may be neglected.

(3) Oxalic acid and diacetic acid evolve carbon monoxide in considerable amounts and are, therefore, to be eliminated—the oxalic acid by treatment with calcium chloride and ammonia, and the diacetic acid by boiling with mineral acid.

¹ Meissner, R., *Biochem. Z.*, 1915, lxxviii, 175.

² Schneyer, J., *Biochem. Z.*, 1915, lxx, 294.

From the statements of Schneyer, the method seemed to fulfil its purpose better than any of the other indirect methods.

The values obtained frequently exceeded those obtained by other methods, so that one thought of the possible presence of substances, other than lactic acid, which were capable of yielding carbon monoxide. Schneyer reported no experiments, but promised the publication of results on normal and pathological urines. We had no opportunity of obtaining the later literature from abroad, and it is possible that Schneyer may in the meantime have recognized the limitations of the method.

Search was started for substances occurring in urine which could survive treatment with calcium chloride and ammonia, and which, when extracted with ether, would yield carbon monoxide.

TABLE I.

Date.	Patient.	Age.	Diagnosis.	Urine in 24 hrs.	Sp. gr.	Lactic acid.	
						Total as created, Schneyer.	Corrected for hippuric acid.
		yrs.		cc.		gm.	gm.
Oct. 17, 1916.....	P.	6½	Endocarditis.	350	1020	0.1942	0.1630
" 31, 1916.....	B1.	8½	"	515	1010	0.5613	0.4945
June 7, 1917.....				815	1018	0.2575	0.1076
May 29, 1917.....	B2.	8	"	450	1020	0.1543	0.0651

Contrary to the statement of Schneyer, hippuric acid was found to give CO, due to the dissociated glycocholl which alone gives CO. The reaction does not proceed molecularly, but 0.1 gm. of hippuric acid was found to give consistently 0.0057 gm. of carbon monoxide. The amount of hippuric acid in the ether extracts of the urine was determined in a few cases by the method of Folin and Flanders.³ From the amounts of hippuric acid found, the carbon monoxide from this source was calculated as lactic acid, and the original values for lactic acid were corrected. In Table I will be seen the values for lactic acid before and after this correction has been made.

It is evident that hippuric acid cannot be neglected offhand.

³ Folin, O., and Flanders, F., *J. Biol. Chem.*, 1912, xi, 257.

Succinic acid liberates carbon monoxide easily when treated with sulfuric acid. When added to urine which was treated according to the Schneyer method, a decided increase in the amount of carbon monoxide was found. Succinic acid is said to occur in normal urines, and it has been found in pathological exudates (Hoppe-Seyler).⁴ It is not at all unlikely, therefore, that some of the carbon monoxide may be derived from this source at times.

β -Hydroxybutyric acid, which occurs in normal urine of children⁵ yielded a negligible amount of carbon monoxide in agreement with Schneyer's statement. Oxalic acid also yielded no carbon monoxide when added to urine which was treated according to Schneyer's method.

The values for lactic acid in normal urines which are given by indirect methods vary from 6 to 8 mg. per 100 cc. of urine. Ryffel,⁶ who has written a very good review of the work done on the occurrence of lactic acid in urine under normal and pathological conditions, gives 6 mg. per 100 cc. of urine (sp. gr. 1020) as a normal value. Von Fürth⁷ gives 8 mg. per 100 cc. as a normal average, using the bisulfite-iodine titration after clearing the urine with phosphotungstic acid and oxidizing with permanganate. Ishihara⁸ also finds that a value of about 8 mg. per 100 cc. is normal. In non-pathological cases, the Schneyer method gave values very near the values obtained by these indirect methods. Table II shows the values obtained under different conditions by the Schneyer method.

Comparative determinations were made using the method devised by Ryffel,⁹ a brief outline of which follows. 40 cc. of urine to which a known amount of lactic acid has been added are placed in a 500 cc. flask, and to this are added 45 cc. of concentrated sulfuric acid, rapidly through a dropping funnel, while the flask is shaken and cooled. The flask is connected to a steam

⁴ Hoppe-Seyler, F., *Z. physiol. Chem.*, 1894, xix, 476.

⁵ Veeder, B. S., and Johnston, M. R., *Am. J. Dis. Child.*, 1916, xi, 291.

We are indebted for this acid to Professor Shaffer.

⁶ Ryffel, J. H., *Quart. J. Med.*, 1909-10, iii, 221, 413.

⁷ Von Fürth, O., *Wien. klin. Woch.*, 1914, 877.

⁸ Ishihara, H., *Biochem. Z.*, 1913, l, 468.

⁹ Ryffel, J. *Physiol.*, 1909-10, xxxix, p. v.

TABLE II.

Date.	Patient.	Age.	Urine in 24 hrs.	Sp. gr.	Lactic acid.		Remarks.*
					Total excreted, Schneyer.	Per 100 cc. of urine.	
		yrs.	cc.		gm.	gm.	
Mar. 22.....	F1.	10	950	1015	0.0798	0.0084	Orthopedic operation on foot.
" 30-31....			1,135	1017	0.08502	0.0075	
	W.	11	560	1020	0.0554	0.0075	Operation after poliomyelitis.
Feb. 21.....	F2.	6	485	1012	0.03607	0.0062	Pneumonia.
" 29			575	1010	0.1104	0.0192	
	M.	4	110	1020	0.0360	0.0330	Pneumonia, trace of acetone.
Mar. 2.....	S1.	9	520	1027	0.0925	0.0178	Pneumonia, urine very acid with some albumin and acetone.
" 5.....			500	1027	0.1150	0.0230	Pneumonia, urine very acid with some albumin and acetone.
	S2.	8	372	1015	0.0457	0.0123	Pneumonia.
	A.	10	540	1014	0.0313	0.0058	Chorea.
Nov. 1, 1916..	B1.	8½	735	1015	0.2947	0.0401	Endocarditis.
June 14, 1917..			410	1028	0.1683	0.0411	"

* Unless noted, no abnormalities in the urine were found.

generator and a condenser. A gentle current of steam is led into the flask, which is heated to 153–157° for 1 hour, or until 150 cc. of fluid have distilled. The distillate is made alkaline and redistilled. The second distillate is collected in a 100 cc. volumetric flask and compared colorimetrically with a standard prepared by the addition of Schiff's reagent (rosaniline hydrochloride decolorized with sulfurous acid) to a known solution of formaldehyde. I found it necessary to run a control experiment with a known solution of lactic acid to standardize the color produced by the Schiff's reagent with the formaldehyde each time the standard was made. This method shares, for very accurate work, the objections raised by Dehn¹⁰ against all colori-

¹⁰ Dehn, W. M., *J. Am. Chem. Soc.*, 1917, xxxix, 1392.

metric methods. But the criticism of Ishihara⁸ with reference to the loss of acetaldehyde in the distillation was not borne out by experiments in which I used much smaller amounts of lactic acid.

Table III shows that it is possible to determine added lactic acid with the Ryffel method as well as with the Schneyer method.

TABLE III.

Date.	Patient.	Age.	Lactic acid.					Remarks.
			Per 100 cc. of urine.	Per 100 cc. of urine and lactic acid.	Found, added.	Added.		
Ryffel method.								
J an. 7, 1917...	B3.	13	0.0080	0.0200	0.0120	0.0119	Recovered chorea.	
N ov. 14, 1916.	B1.	8½	0.0095	0.0562	0.0467	0.0470	Determination made on ether extract of urine.	
J an. 8, 1917...			0.0100	0.0225	0.0125	0.0119	Endocarditis.	
Schneyer method.								
J an. 7, 1917...	B3.	13	0.0179	0.0298	0.0119	0.0119	Recovered chorea.	
N ov. 14, 1916...	B1.	8½	0.0337	0.0568	0.0231	0.0230	Endocarditis.	
J an. 8, 1917...			0.0305	0.0440	0.0135	0.0119	"	

After the correction had been made for hippuric acid as previously described, the values for lactic acid as determined by the Schneyer method were higher than those obtained with the Ryffel method, as in the following examples.

Date.	Patient.	Age.	Urine in 24 hrs.	Sp. gr.	Total lactic acid found in 24 hrs. by Schneyer's method.	After correction for hippuric acid. Ryffel and Schneyer.	Remarks.
1917		Yrs.	cc.		gm.	gm.	
June 7.....	B1.	8½	815	1018	0.2575	R. 0.0509 S. 0.1076	Endocarditis.
May 29.....	B2.	8	420	1020	0.1543	R. 0.0180 S. 0.0651	"

The excess carbon monoxide calculated as lactic acid is therefore due in part to substances¹¹ other than hippuric acid.

TABLE IV.
Comparative Results Obtained by the Two Methods.

Date.	Patient.	Age.	Urine in 24 hrs.	Sp. gr.	Lactic acid.		Remarks.
					Total. S., Schneyer. R., Ryffel.	Per 100 cc. of urine.	
		Yrs.	cc.		gm.	gm.	
Jan. 7, 1917.	N.	1	350	1025	S. 0.0315 R. 0.0245	S. 0.0090 R. 0.0070	Recovered bronchitis.
Dec. 12, 1916.	S3.	1½	420	1018	S. 0.0811 R. 0.0126	S. 0.0193 R. 0.0030	" "
Mar. 23.....	F3.	6	750	1010	S. 0.0570 R. 0.0150	S. 0.0076 R. 0.0020	Recovered cystitis.
" 29.....			532	1016	S. 0.0646 R. 0.0063	S. 0.0122 R. 0.0012	" "
	S4.	7	840	1015	S. 0.0747 R. 0.0184	S. 0.0089 R. 0.0022	Hemophilia.
Nov. 14.....	B1.	8½	885	1010	S. 0.2982 R. 0.0840	S. 0.0337 R. 0.0095	Endocarditis.
May 23.....	B2.	8	375	1016	S. 0.1203 R. 0.0187	S. 0.0321 R. 0.0050	"

It is perfectly clear, even from the relatively small number of our experiments, that the method of Schneyer cannot be applied to the quantitative determination of lactic acid in urine. But the method is of unquestionable value, indicating the excretion of substances belonging to a group capable of yielding carbon monoxide under the conditions of the experiment.

¹¹ Camphor glycuronic acid (not crystalline) prepared according to the method described by O. Schmiedeberg and H. Meyer (*Z. physiol. Chem.*, 1879, iii, 422) gave CO, while camphor alone did not.

STUDIES OF FOOD UTILIZATION.

I. THE UTILIZATION OF CARBOHYDRATE ON RELATIVELY HIGH AND LOW CEREAL DIETS.

By ZELMA ZENTMIRE AND CHESTER C. FOWLER.

(From the Laboratory of Physiological Chemistry, Department of Chemistry, Iowa State College, Ames.)

(Received for publication, August 18, 1917.)

INTRODUCTION.

While there are on record many experiments to determine the utilization of protein, carbohydrate, and fat from various sources, few seem to have been made to determine the utilization of these constituents when ingested in *varying quantities*. There seem to be no data on the comparative utilization of varying amounts of these food principles when derived from cereals in some form other than white bread. The present study was undertaken to ascertain how much if any difference occurs in the utilization of cereal protein and cereal carbohydrate when ingested in varying amounts in the form of thoroughly cooked "cream of wheat." The data on protein utilization will be presented in a later paper.

Carbohydrates Present in Wheat Farinas.

Since the utilization of carbohydrate depends to some extent upon its nature, a knowledge of the forms present in the wheat farinas, in which class cream of wheat belongs, is not without importance. Ishida and Tollens (1) reported 6.93 per cent pentosans and 1.72 per cent methyl pentosans in wheat. Teller (2) closely agreed in reporting 7.43 per cent pentosans in wheat. He also found 0.0 per cent dextrose, 1.46 per cent sucrose, and 53.65 per cent starch and undetermined carbohydrate in ripe wheat. The bran contains most of the pentosans and crude fiber. That the original sugars in wheat meal are dextrose and sucrose, which are present in small amounts (0.1 to 0.4 per cent and 1.0 to 1.5 per cent respectively) has also been determined by von Liebig (3).

Utilization of Carbohydrates.

According to Cammidge (4) and also to Thomsen (5) cane sugar is completely absorbed. London and Polowzowa (6) reported as the result of experiments upon dogs with fistulas that in the lower ileum glucose and sucrose are completely absorbed, and starch paste is 93.3 per cent absorbed. Dry starch to the extent of 21.9 per cent was found to pass into the large intestine. Fofanow (7) found the normal gastrointestinal tract capable of digesting and absorbing, equally well, raw or boiled starch from wheat, oats, and rice. The quantity of carbohydrate in the feces after the ingestion of these raw starches was small. On the other hand, the extent of utilization of carbohydrate was found by Ehrmann and Wolff (8) to be dependent upon the amount of carbohydrate fed, the state of division, and especially upon the thoroughness of cooking. These experiments were made upon dogs, and the feces were quantitatively analyzed for starch and easily hydrolyzed cellulose. Harcourt and Fulmer (9) say wheat and oat meals boiled 20 minutes are almost as fully digested as those cooked 8 hours.

Schneider (10) states that pentosans of apple marc are digested by man to the extent of 88.7 per cent, but that bacteria of the alimentary tract, and not enzymes are responsible for their digestion. Any cellulose present in such finely divided form as in wheat farinas would probably be utilized (11, 12, 13).

The carbohydrates of milk, of wheat bread, and of a simple mixed diet are particularly well digested (14). It is to be noted that the work of the several investigators indicates that varying amounts of carbohydrate up to 670 gm. in the form of wheat bread are practically equally well utilized. Harcourt (15) determined the coefficient of digestibility for many cereal foods. Healthy young men were the subjects, and the periods were 4 days in length. The diets consisted of the breakfast food with cream and sugar. Typical results follow in Table I (15).

TABLE I.
Utilization of Carbohydrates on Cereal Diets.

	No. of experiments.	Total carbo- hydrate digested.	Cereal carbo- hydrate digested, calculated.
		<i>per cent</i>	<i>per cent</i>
Wheat farinas.....	11	98.2	98.8
“ germ.....	3	98.5	98.8
Rolled wheat..	3	95.2	94.8
Granulated oats.....	6	97.6	98.1
Rolled oats.....	10	98.2	98.4

The data on the whole point toward a loss of between 1 and 2 per cent of the carbohydrate on a diet of wheat or oat cereal with cream and sugar.

The fact that carbohydrate other than that of the cereal was ingested makes uncertain the utilization of the cereal carbohydrate, although the latter has been estimated.

EXPERIMENTAL.

The subject was a mature normal young woman weighing at the time of the experiment approximately 49 kilos and engaged in moderately active physical and mental work pertaining to teaching and laboratory routine. The experiment covered a period of 3 weeks divided as follows: 5 days each of lower and higher cereal diets; 2 days each of nitrogen-free lower and higher starch diets; and preliminary and intermediate periods of 2 days each in which a simple mixed diet was ingested. This diet was calculated to furnish an energy value of 2,300 calories per day, 13.5 per cent of this being furnished by protein. The protein was estimated to yield 12.5 gm. of nitrogen. No analysis of the simple mixed diet was made, the calculation being based on data obtained from Atwater and Bryant (16). An adequate supply of water was taken daily and was made uniform throughout the experiment (17).

Preliminary trials with the experimental diets were made to ascertain the quantities that could be ingested per day. It was learned that in order to keep the protein content of the cereal diets normal, a pure protein well utilized, such as casein (18), would have to be added, and also that difficulty was likely to be encountered in maintaining a sufficient number of calories in the diets. Probably the majority of subjects would not find the necessary quantities of sugar and butter fat distasteful, but this subject ordinarily used comparatively little butter and sugar. In order to make possible the ingestion of adequate quantities of fat, the amounts estimated to be necessary for the higher cereal and the higher starch diets were cooked in the cereal and in the starch pudding.

Cream of wheat was the cereal chosen, because of its homogeneity and because it was liked by the subject. For the starch pudding, the best quality of cornstarch was used.

Cereal and starch in sufficient quantities for the entire experiment were boiled a few minutes on the stove, then placed in a large fireless cooker over night. This method resulted in uniform, thoroughly cooked products which were preserved by cold without freezing. A known weight of butter fat was cooked in each food, and from the weight of the cold product the percentage of fat was calculated with sufficient accuracy to estimate its fuel value. A preliminary analysis of cooked cream of wheat had shown its fat content to be negligible. This result agrees with the low fat content, 2.57 per cent of the ripe wheat grain reported by Teller (2). The starch pudding made with water was flavored with lemon juice, but like all other nitrogen-free diets it was unpalatable. To obtain fat free from nitrogen, butter was melted on a water bath at 45°C., and the clear fat poured off not too closely. To increase the palatability of the cereal diets, whole milk to which small amounts of cream had been added was

taken with each meal. Sufficient rich milk for an entire period of 5 days was preserved in the same manner as the cereal and starch pudding.

From the results of the food analyses, the amount of nitrogen contained in the quantity of cooked cereal that could be ingested daily for the lower cereal diet (considering that this quantity would have to be doubled for the higher cereal diet) was ascertained. Sufficient milk casein was added to make the daily total nitrogen content approximately 9 gm. Butter fat and cane sugar were added to complete the fuel value to 2,000 calories.

The higher cereal diet differed from the lower cereal diet only in that it contained twice as much cereal carbohydrate and protein, and in the amounts of milk sugar and milk fat, due to differences in the percentage composition of the milk used in the two periods. The amount of nitrogen furnished by the milk of the two cereal diets was kept constant. The moisture content of the simple mixed and the experimental diets was kept constant.

For the lower starch diet, as many grams of the pudding were ingested daily as could be, and still be doubled in amount in the higher starch diet. As much butter fat and cane sugar as possible were added, since at best the fuel value of the diet would be none too high. The higher starch diet differed only in that it contained twice as much starch.

The meals of any one period were exactly alike. 3 gm. of agar-agar, which is practically indigestible (19) but stimulates peristalsis, were included in each cereal meal. The milk, cereal, and agar-agar were warmed over hot water. The cereal diets were not unpalatable, except that on the first day the casein caused nausea, and only a part of the usual meal could be ingested. This difficulty arose through an attempt to take the casein in the warm cereal. When suspended in water, the casein was taken with ease. Mendel and Fine (20) have shown that while certain wheat preparations caused intense nausea in man, and necessitated forced feeding in dogs, they were thoroughly digested.

The starch pudding was taken cold. The sugar and butter fat were made into a candy with a portion of the water allowed for the meal. Even then they were taken with difficulty. Because of headache and nausea, induced probably by worry over the experiment, no dinner could be taken the second day of the lower starch period, and the starch pudding had to be omitted for supper of the same day. Agar-agar was nauseating, either dry or in water, and very little was taken with the starch diets. In spite of the headache and nausea mentioned, on the whole the condition of the subject was improved during the 3 weeks of the experimental periods.

The feces of one period were separated from those of another by the ingestion of carmine or charcoal capsules with the first meal of each period. Although feces cannot be separated satisfactorily for periods of less than 2 days' duration, there was no difficulty in making the separation for periods of 2 days in this study, which may have been because of

the comparatively light color of the stools resulting from diets containing little or no meat. The feces were collected in daily samples, weighed at once by difference, preserved with thymol in air-tight cans, and frozen for about a month until analyses could be made. This method of preservation has been found to be satisfactory (21). Analyses were made upon the moist daily samples unless at the beginning or close of a period the portion of the stool from an experimental diet was too small in amount to be analyzed separately.

Methods of Analysis.

All analyses were made in duplicate according to the following methods upon approximately the weight of samples specified.

A. Moisture.

1. Cooked cereal and starch pudding; official method (22); 3 gm. samples.
2. Milk; open dish method (23); weighed 5 cc. samples.
3. Feces; as described by Lohrlich (24); 2 gm. samples.

B. Fat.

1. Cooked cereal containing no added butter fat; Soxhlet method (25); 2 gm. samples.
2. Milk; Babcock method (23); 17.6 cc. samples.

C. Carbohydrates.

1. Reducing sugar in cooked cereal; Munson and Walker method (23); 10 gm. samples.
2. Total carbohydrates in cooked cereal and in starch pudding; official method (22); 10 gm. samples.
3. Milk sugar; Fehling process with the O'Sullivan-Defren gravimetric method (23); 25 cc. samples.
4. Feces; Strasburger method (24); 4 gm. samples.

D. Nitrogen.

1. Cooked cereal; Gunning-Arnold-Dyer modification (25) of the Kjeldahl method; 8 gm. samples.
2. Casein; modified Kjeldahl method (25); 0.35 gm. samples.
3. Milk; " " " 5 cc. "
4. Feces; " " " 2 gm. "

Butter fat present in the cooked cereal and in the starch pudding was removed by repeated washings with ether before car-

TABLE II.
Dietaries for the Periods.

		Weight.	Carbohydrate.	Fat.	Water.	Nitrogen.	Fuel value.
		gm.	gm.	gm.	gm.	gm.	calo.
Lower cereal, diet, 5 days.	Cooked cereal....	150.0	16.5	14.5	115.9	0.5	
	Milk.....	128.8	5.9	8.6	96.4	0.7	
	Sucrose.....	32.0	32.0	0.0	0.0		
	Butter fat.....	18.5	0.0	18.5	0.0		
	Water.....	340.0			340.0		
Total per meal.....			54.4	41.6	552.3		
Daily averages.....			158.6*	121.4*	1,639.6*	8.7*	2,000*
Higher cereal diet, 5 days.	Cooked cereal....	300.0	33.0	29.0	231.8		
	Milk.....	123.4	5.7	9.0	103.8		
	Sucrose.....	32.0	32.0	0.0	0.0		
	Butter fat.....	3.0	0.0	3.0	0.0		
	Water.....	230.0			230.0		
Total per meal.....			70.7	41.0	565.6		
Daily averages.....			212.1	123.0	1,696.8	10.4	2,250
Lower starch diet, 2 days.	Starch pudding..	100.0	16.7	11.2	72.0		
	Butter fat.....	13.0	0.0	13.0	0.0		
	Sucrose.....	70.0	70.0	0.0	0.0		
	Water.....	490.0			490.0		
Total per meal.....			86.7	24.5	562.0		
Daily averages.....			208.5*	55.5*	1,614.0*	0.0	1,350*
Higher starch diet, 2 days.	Starch pudding..	200.0	33.5	22.4	144.0		
	Butter fat.....	1.0	0.0	1.0	0.0		
	Sucrose.....	70.0	70.0	0.0	0.0		
	Water.....	420.0			420.0		
Total per meal.....			103.5	23.4	564.0		
Daily averages.....			310.5	70.2	1,692.0	0.0	1,900

* Allowances have been made for failure to ingest a part of the meals, as mentioned.

TABLE III.
Fecal Analysis and per Cent of Digestibility of Carbohydrate.

Period.	Fecal analysis.			Carbohydrate intake.		Percentage digestibility.	
	Weight.	Dry matter.	Starch.	Total.	One carbo- hydrate.	Total carbo- hydrate.	One carbo- hydrate.
	gm.	gm.	gm.	gm.	gm.		
Lower cereal, 5 days.	84.1	18.6	1.4				
	106.6	20.1	1.7				
	80.8	17.8	1.1				
	79.2	19.6	1.5				
Total.....	350.7	76.1	5.7				
Daily average.....	70.1	15.2	1.1	158.6	48.2†	99.3	97.7†
Higher cereal, 5 days.	81.2	18.2	1.9				
	90.4*	23.4	1.9				
	61.9	15.5	0.7				
	43.4*	11.5	0.6				
Total.....	277.3	68.6	5.1				
Daily average.....	55.5	13.7	1.0	212.8	99.0†	99.5	99.0†
Lower starch, 2 days.	69.7*	18.1	1.0				
Daily average.....	34.9	9.1	0.5	208.5	33.5†	99.8	98.5†
Higher starch, 2 days.	80.7*	21.2	2.8				
Daily average.....	40.4	10.6	1.4	310.4	100.4†	99.6	98.6†

* Stools of 2 days were analyzed together.

† Cereal carbohydrate (assuming sucrose and lactose to be 100 per cent digestible).

‡ Starch (assuming sucrose and lactose to be 100 per cent digestible).

bohydrate determinations were made. As might be expected from the composition of wheat (2, 3), only traces of reducing sugar were found in the cooked cereal. Fecal analyses were made upon moist samples, to reduce error (21, 24). Instead of using dry feces with 2 per cent HCl as directed for the carbohydrate determination, moist samples were used with 2.2 per cent HCl to allow for moisture present.

In Table III are tabulated the results of fecal analyses and the *coefficients of digestibility*. Throughout the study with the exception of 1 day following the lower starch diet period, normal movement of the bowels took place daily, usually in the morning. The fact that little agar-agar was taken with the last two experimental diets accounts for the slightly reduced amount of dry matter in the feces of these periods. Microscopical examination failed to show the presence of unruptured starch grains in any of the stools.

There was no significant change in body weight throughout the experiment.

CONCLUSIONS.

In so far as conclusions may be drawn from one experiment, upon one individual, with one cereal, the data secured seem to justify the following conclusions.

1. The utilization of total carbohydrates of a diet consisting largely of cereal is above 99 per cent.
2. The carbohydrate is as completely utilized with one quantity as another of cereal in the diet, even when the cereal is taken in larger amounts than are found in the average dietary.
3. Assuming that sucrose and lactose are completely digested and absorbed, the utilization of the cereal carbohydrate is still high (97.7 to 99.0 per cent).
4. Assuming that sucrose is completely utilized, the starch of the starch diets is utilized to the extent of over 98 per cent.
5. Monotony and unpalatability of diet have little or no effect upon the ultimate utilization.

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METHODS FOR APPROXIMATING THE RELATIVE TOXICITY OF COTTONSEED PRODUCTS.

By FRANK E. CARRUTH.

(From the Laboratory of the North Carolina Agricultural Experiment Station,
West Raleigh.)

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During investigations of the cause of cottonseed meal poisoning or cottonseed meal "injury" at the North Carolina Experiment Station it was shown¹ that in general the relative toxicity of cottonseed meals varied with the extent to which the raw cottonseed kernels are cooked in the oil mill.

The raw kernels are highly toxic to animals, owing to the fact that they contain about 0.6 per cent gossypol, a phenolic plant pigment, which has been shown by Withers and Carruth² to possess markedly toxic properties. On cooking with moist heat as in the hot pressing processes preparatory to expressing the oil, the glands containing gossypol are disintegrated and the gossypol is spread over the surface of the seed tissue, and apparently undergoes a change which is assumed to be a partial oxidation of the gossypol molecule. The change takes place under suitable conditions very quickly so that some meals which have been cooked only 20 to 30 minutes are not markedly toxic for rats.

This cooking process causes the ether-soluble and oil-soluble gossypol to be changed to a substance which *in the meal* is no longer ether- and oil-soluble, possibly because it is in some way chemically combined with some constituent of the meal (protein).

Its presence in considerable amounts may be demonstrated in ether-extracted cottonseed meal by treating the meal with hot alcoholic potash. The supernatant liquid contains the substance which, like gossypol, soon oxidizes with the production of a beautiful blue color. The substance is much less toxic than

¹ Withers and Carruth, unpublished work. See also Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxix, 289.

² Withers, W. A., and Carruth, F. E., *J. Agric. Research*, 1915, v, 261.

gossypol and, for convenience, we have termed this much less toxic and less soluble form "D" gossypol.

If the highly toxic gossypol itself is present in the meal, this may be extracted by ether or detected by the following microscopic method.

1. *H₂SO₄ Test for Unchanged Gossypol.*—A bit of the meal is sprinkled on a glass slide and touched with a drop of concentrated sulfuric acid. The result is observed immediately through the low power of a microscope. If numerous red areas appear where the acid touches the more or less broken up "glands" it is an indication of the presence of toxic unchanged gossypol. Thoroughly cooked meals, and cottonseed flour show very few and very small red areas. Meals cooked with insufficient moisture or for too short a time show the presence of relatively many red areas. The red color is characteristic of gossypol in strong sulfuric acid.

2. *Quantitative Determination of Gossypol Present in Cottonseed Meal,—Aniline as Precipitant.*—This method, which is a measure of the amount of gossypol *as such* present in the meal, is based on the fact that it is easily extracted by ether, together with the oil. Gossypol forms a very sparingly soluble compound³ with aniline, which separates from the oily extract and which may be filtered out and weighed. The insoluble "D" gossypol is not extracted by ether.⁴

Procedure.

Enough cottonseed meal (preferably 200 gm. or more of cottonseed meal) is extracted 2 to 3 hours with ether so as to give 5 to 10 gm. of oil. The meal may be simply percolated, or shaken with ether in a flask. The extract is evaporated to small volume and filtered. Aniline is added (about 10 per cent of the weight of the extract) and the mixture warmed on the water bath and

³ This compound was first made by Dr. J. T. Dobbins in this Station.

⁴ The presence of nearly 1 per cent of "D" gossypol in well cooked cottonseed meal may be shown by extraction of *ether-extracted meal* with warm aniline. The aniline salt separates out on cooling the filtered solvent. Since well cooked meals rarely show the presence of gossypol *as such*, it is evident that this "D" gossypol is responsible for the "cottonseed meal poisoning" of swine. Complete proof of this will soon be published.

TABLE 1.
*Comparison of the H₂SO₄ Test for Gossypol and the Percentage of Gossypol
with the Toxicity of the Cottonseed Product.*

No.	Cottonseed product.	Percentage of diet.	H ₂ SO ₄ test (red areas).	Aniline method (percentage of gossypol).	Effects on rats (gain or loss in weight).
1	Raw kernels.	10-60	Maximum plus.	0.63	Very toxic.
2	" " ether-extracted.	50	Trace.	—	+40 to 50 per cent in 30 days.
3	Kernels cooked 5 min.	70	Maximum plus.	0.62	-28 per cent in 5 days; fatal.
4	Kernels cooked 10 min.	70	Many.	0.24	-18 per cent in 10 days; fatal.
5	Kernels cooked 20 min.	—	Disappearing.	0.10	No gain in 37 days.
6	Kernels cooked 28 min.	70	"	0.07	-23 per cent in 37 days
7	Kernels from pre-heaters of cold pressing mill.	—	Maximum plus.	0.57	Not fed.
8	Meal from same.	50	Few small areas.	0.02	+3 per cent in 8 days.
9	Oil " "	14	—	1.50	-30 per cent in 14 days; fatal.
10	Kernels cooked 2 hrs.	—	—	No ppt.	
11	Meal from same.	50	Very few and small.	" "	+8 to 12 per cent in 30 days.
12	Oil " "	14	—	" "	Non-toxic.
13	Hot pressed meal "T" (dry conditions).	—	Many.	0.074	-13 per cent in 14 days.
14	Laboratory cold pressed meal.*	—	Maximum.	—	Toxic.*
15	Oil from same.	—	—	—	Non-toxic.*
16	Actually cold pressed meal from hydraulic oil mill press.	—	Very many.	0.49	Not fed.
17	Oil from same.	—	—	No ppt.	" "

* This product was a sample secured from Dr. Osborne. The effects of feeding this product and the cold pressed oil are described by him.¹ It is interesting to note how the results secured by simple pressure differ from the grinding pressure of the screw press of a commercial "cold pressing" mill (compare Nos. 7, 8, and 9 of this table with Nos. 14 to 17 inclusive).

then set aside to stand. The more gossypol there is present, the quicker will be the appearance of a yellow microcrystalline precipitate of the aniline-gossypol compound. This substance appears to be the dianiline salt of gossypol, $\text{—C}_{30}\text{H}_{28}\text{O}_9 \cdot 2\text{C}_6\text{H}_5\text{NH}_2$, and the factor 0.74 is used to convert the weight of the precipitate to weight of gossypol. If after several days' standing no precipitate appears, as has often been the case, it may be assumed there is little or no gossypol present as such in the meal. The more toxic meals give a precipitate of the aniline-gossypol compound which is filtered out in a tared Gooch crucible, washed with a mixture of ether and petroleum ether (about 1:2), and then washed clean with petroleum ether, dried at 100°C ., and weighed.

The aniline-gossypol compound is not completely insoluble in an oily mixture but it has been found possible to recover more than 90 per cent of 0.5 gm. of gossypol dissolved in 50 cc. of purified cottonseed oil by this method.

The relation between some observations and determinations, and the toxicity of some cottonseed products is summarized in the table.

Attention is called to the fact that the commercial cold pressed meal is not apt to be more toxic than the hot pressed meal. The reason for this appears on examination of the "cold pressed" oil which was found to contain about three-fourths of the total gossypol of the seed. While hot pressing processes vastly reduce the toxicity of the cottonseed by causing a chemical change, the so called cold pressing process causes a similar reduction by squeezing most of the toxic gossypol into the oil. (The gossypol is of course entirely removed in the refining process.) Most samples of hot pressed oil fail to show more than a trace of gossypol, confirming the observation made previously; namely, that practically all the partially oxidized product remains in the hot pressed meal.

The author acknowledges the courtesy shown him by the managers of the oil mills in North Carolina, from which most of the above samples were obtained.

Several of the analyses in the table were made by Dr. L. F. Williams and Dr. John Dobbins of the North Carolina State College of Agriculture, whom the author desires to thank.

THE NUTRITIVE VALUE OF THE SOY BEAN.

By AMY L. DANIELS AND NELL B. NICHOLS.

(*From the Department of Home Economics, University of Wisconsin, Madison.*)

(Received for publication, August 17, 1917.)

A consideration of the chemical composition of the soy bean¹ (*glycine hispida*) suggests that it may be a food of considerable value in the human dietary. It averages a high percentage of protein (36.5 per cent) and fat (17.5 per cent) as well as considerable inorganic material (5.3 per cent). Its digestible carbohydrate (12 per cent) is lower than that of many of the leguminous seeds, and is largely in the form of sucrose rather than starch.² At the present time, however, chemical analysis cannot be considered an adequate method of determining the nutritive efficiency of a given food. By such means investigators are able to find out the nature and relative amounts of the chemically familiar foodstuffs, fat, carbohydrates, protein, and inorganic material; but since the optimum amounts of the various amino-acids for physiologic well-being have not yet been determined, nor the chemical nature of the food accessories ascertained, in addition to chemical determinations biologic tests must be made.

In a preliminary investigation rats fed the yellow soy bean (*Ito San*) failed to grow; therefore such additions were made as would lead to a knowledge of the food value of this legume. In all cases the beans were cooked in water until tender (from 30 to 40 minutes) under 15 pounds' pressure. When starch was used in the ration, this was incorporated with the other materials, and boiled from 3 to 5 minutes.

¹ Henry, W. A., and Morrison, F. B., *Feeds and Feeding*, Madison, 1915, 656.

² Pott, E., *Handbuch der tierischen Ernährung und der landwirtschaftlichen Futtermittel*, Berlin, 1907, ii, 540.

The influence of the addition of a suitable inorganic mixture to the soy bean ration is shown by those animals (Chart I) which when partly grown were fed cooked soy beans for periods of 7 to 13 weeks respectively. During this time the weight remained almost stationary and the females failed to reproduce. When these animals were placed on a ration consisting of soy beans 60, lard 16, cornstarch 18.7, and a salt mixture 6.3 per cent which made the inorganic portion of the ration comparable to that of milk, they gained in weight and within 5 and 6 weeks the females reproduced. In two instances second pregnancies followed at short intervals. The young were successfully suckled in all cases. These results are in accord with work of McCollum, who has found that the principal cause for the failure of rats to grow on diets made up of seeds consists in the amount and character of the inorganic complex of the diet. The addition of those elements, especially calcium, chlorine, and sodium, in which the soy bean is low,⁵ made them a more nearly perfect food.

But the fact that adult animals on the soy bean inorganic mixture were under sized, while the young were not only smaller at birth (3 gm.) but the litters were in all cases smaller, consisting of two and three each, points to the conclusion that the soy bean is somewhat lacking in other respects. Furthermore, when the young (Chart II) were placed on the diet of the adults, they passed through a period, lasting from 4 to 5 weeks, in which there was little gain in weight. The mortality of these animals during this period was high and all of the second litters succumbed. Not one of the second generation of rats upon this ration has reproduced; indeed, at the end of the fourth month if not before, the young animals, with one exception, have died, unless the fat-soluble food accessory was added to the diet. Even then in some instances the addition of butter fat failed to prevent nutritive deficiencies.

³ There is considerable variation in the protein content of soy beans, depending upon conditions of soil and climate. Determinations of the protein content of the *Ito San*, made in the Department of Agronomy, University of Wisconsin, show a seasonable variation between 30 and 45 per cent in the same strain. The beans used in this investigation contained 31.2 per cent of protein ($N \times 6.25$).

⁴ McCollum, E. V., *J. Am. Med. Assn.*, 1917, lxviii, 1379.

⁵ Forbes, E. B., *Ohio Agric. Exp. Station Bull.* 207, 1909.

the comparatively light color of the stools resulting from diets containing little or no meat. The feces were collected in daily samples, weighed at once by difference, preserved with thymol in air-tight cans, and frozen for about a month until analyses could be made. This method of preservation has been found to be satisfactory (21). Analyses were made upon the moist daily samples unless at the beginning or close of a period the portion of the stool from an experimental diet was too small in amount to be analyzed separately.

Methods of Analysis.

All analyses were made in duplicate according to the following methods upon approximately the weight of samples specified.

A. Moisture.

1. Cooked cereal and starch pudding; official method (22); 3 gm. samples.
2. Milk; open dish method (23); weighed 5 cc. samples.
3. Feces; as described by Lohrlich (24); 2 gm. samples.

B. Fat.

1. Cooked cereal containing no added butter fat; Soxhlet method (25); 2 gm. samples.
2. Milk; Babcock method (23); 17.6 cc. samples.

C. Carbohydrates.

1. Reducing sugar in cooked cereal; Munson and Walker method (23); 10 gm. samples.
2. Total carbohydrates in cooked cereal and in starch pudding; official method (22); 10 gm. samples.
3. Milk sugar; Fehling process with the O'Sullivan-Defren gravimetric method (23); 25 cc. samples.
4. Feces; Strasburger method (24); 4 gm. samples.

D. Nitrogen.

1. Cooked cereal; Gunning-Arnold-Dyer modification (25) of the Kjeldahl method; 8 gm. samples.
2. Casein; modified Kjeldahl method (25); 0.35 gm. samples.
3. Milk; " " " 5 cc. "
4. Feces; " " " 2 gm. "

Butter fat present in the cooked cereal and in the starch pudding was removed by repeated washings with ether before car-

at an early age on the diet in which the only source of fat-soluble A has been the 50 per cent of soy bean.

In connection with another investigation, it happened that at the same time that these animals were placed on the soy bean ration, four other animals of the same litter were placed on a diet of purified food substances, otherwise adequate but freed in so far as possible from fat-soluble A⁸ (Chart VI, Rats 35, 36, 37, and 38). The difference in the growth of these two groups of animals, those receiving no fat-soluble A and those receiving a diet containing 50 per cent of soy beans to which no butter fat was added, leaves little doubt that the soy bean contains a fairly liberal amount of the fat-soluble food accessory. Three of the animals on the purified ration grew at about half the normal weight for a period of 4 weeks, then lost weight more or less rapidly. The addition of butter fat was followed by a rapid gain in weight. The animals on the soy bean ration grew at very nearly the normal rate. In this connection it is interesting to note that McCollum found that the extracted oil of the soy bean was quite free from this material.⁹

The question of the possible storage of the fat-soluble food accessory has attracted considerable attention, as well as the relative amounts needed for different periods of the life cycle. From our present study it seems probable that not only can the animal store the fat-soluble material, but that the young need considerably more of it than adults. A diet which was apparently adequate for maintenance and even reproduction in the adult was found to contain too small an amount of the fat-soluble food accessory for the development of the young of mothers fed a limited supply of this material. The addition of butter fat made the ration more nearly perfect for these growing animals (Chart II). Young animals (Chart IV) produced and suckled by

⁸ The diet consisted of 28 per cent purified casein, 27 lard, 32 corn-starch, 5 agar, 8 per cent mineral salts comparable to those in milk, and water-soluble extract from wheat embryo. The casein was prepared by acidifying skimmed milk with acetic acid, filtering the precipitate, and washing for 24 hours in running water. The washed casein was then redissolved in dilute sodium hydroxide, again precipitated with acetic acid, and washed 48 hours in running water.

⁹ McCollum, Simmonds, and Pitz, *Am. J. Physiol.*, 1916, xli. 61.

mothers having a liberal supply of the fat-soluble food accessory appear to be able to withstand a shortage of this material during their most rapidly growing period much better than those animals whose mothers received considerably less. The plausible explanation for this marked difference in the behavior of these two groups seems to be that in the one case the young had been furnished a liberal supply during the gestation and suckling period, which was stored, and therefore a food carrying somewhat less than the optimum amount supplied enough for their needs over a considerable period, whereas those young which were given the minimum amount during infancy were unable to survive on a similar ration.

The value of the protein of the soy bean has been demonstrated in all of our experiments. That animals fed rations containing 15.6 and 18.7 per cent of protein obtained solely from the soy bean have grown normally and in the latter case have produced successive litters of young, which in turn have reproduced, is sufficient evidence that the protein of the soy bean fulfills all physiologic requirements. The protein of the soy bean appears to be quite as valuable as the casein of milk. These findings are somewhat surprising in view of the fact that the protein of other legumens, namely, peas and white beans, has been found wanting.^{4, 6} Our results, however, are comparable to those of Osborne and Mendel,¹⁰ who report successful growth with rats on rations in which glycinin, the principal protein of the soy bean, was used.

Within the last few years investigators have conducted numerous feeding experiments showing that when the water-soluble accessory material is lacking in a dietary, there is no growth, and abnormal physiologic conditions such as beri-beri and polyneuritis occur.¹¹ Since none of the rats in this investigation has presented any of these symptoms, it is logical to conclude that the soy beans, the only possible source of water-soluble B in these rations, contain a considerable amount of this material. Even when soy beans formed as low as 50 per cent of the ration, there was sufficient supplied for the animals under investigation.

¹⁰ Osborne, T. B., and Mendel, L. B., *Carnegie Institution of Washington, Publication 156*, 1911, ii, 121.

¹¹ McCollum, E. V., and Kennedy, C., *J. Biol. Chem.*, 1916, xxiv, 491.

An economic consideration of the yellow soy bean leads to the conclusion that it is one of the most valuable of the leguminous seeds. It contains a high percentage of a physiologically good protein, a considerable amount of energy-yielding material in the form of fat and carbohydrate, and a fairly liberal supply of the fat-soluble food accessory, as well as of the water-soluble growth determinant.

In order to make the soy bean a more nearly complete food, suitable inorganic material, consisting principally of sodium chloride and calcium compounds, needs to be added. In Japan and China this bean has been used extensively as human food for many years. In America, however, it has only recently been introduced into the human dietary, although it has been incorporated in rations for farm animals in certain sections of the country.

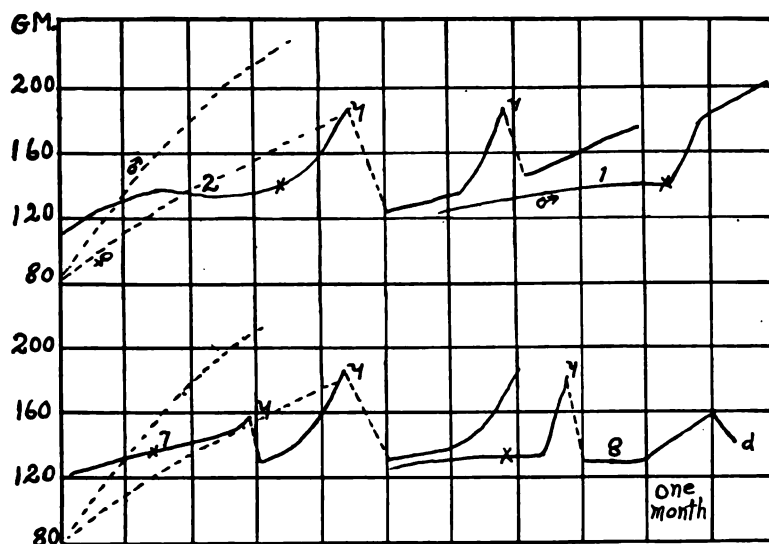


CHART I. Curves of growth of rats fed cooked soy beans. The change to Ration I (indicated by X), containing soy beans, and a suitable mineral mixture, was followed by an increase in weight and reproduction.

Ration I.		Minerals per 100 Gm. of Mixture.	
Soy beans.....	60	NaCl.....	0.838
Lard.....	16	K ₂ HPO ₄	2.26
Cornstarch.....	17.7	CaSO ₄ ·H ₂ O.....	0.015
Minerals.....	6.3	CaCl ₂	0.87
		Ca lactate.....	2.39

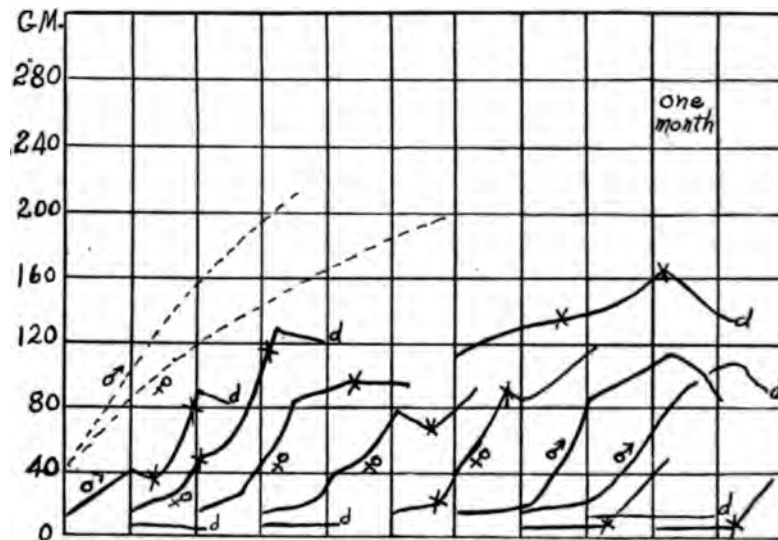


CHART II. Immediately following the suckling period the animals were fed Ration I. The mothers of these animals were similarly fed during the gestation and suckling period. The change to Ration II (indicated by \times), which included 5 per cent butter fat, produced marked improvement in many cases. The removal of the butter fat (indicated by the second \times on the curve) was followed by loss in weight and death in all but one case.

Ration II.

Soy beans.....	60
Lard.....	11
Butter fat....	5
Cornstarch....	17.7
Minerals as in Ration I....	6.3

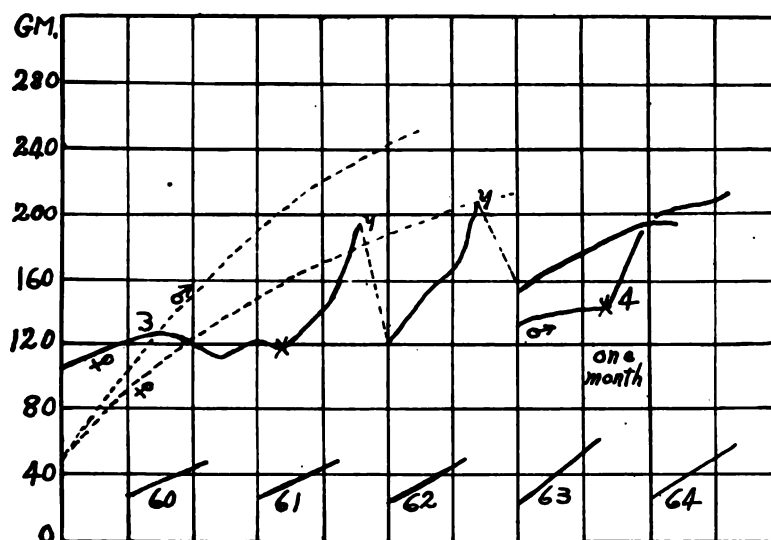


CHART III. The rats in this group were fed cooked soy beans. The change to Ration II (indicated by X), which included a suitable salt mixture and 5 per cent butter fat, was followed by marked improvement and reproduction. The young were unusually large animals, and the litters were also large, consisting of eight and nine each. The young animals have passed through the period of retarded growth which is usual on a bean ration.

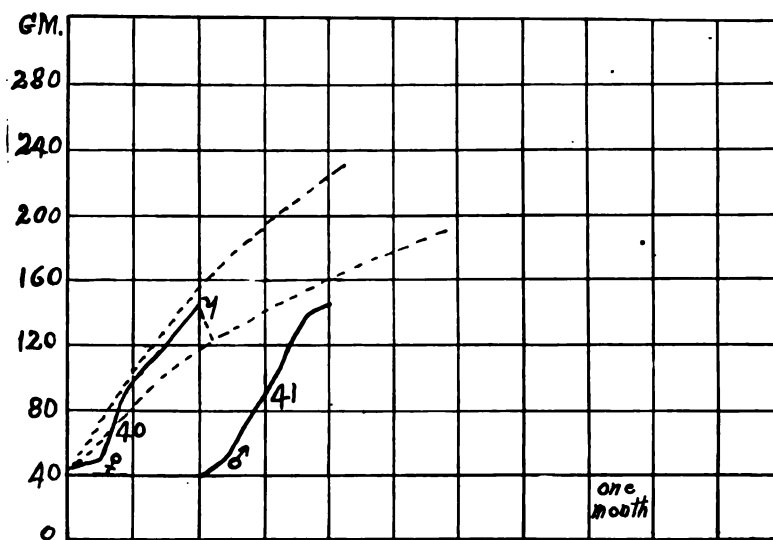


CHART IV. Curves of growth of rats fed Ration III, which included no butter fat. The diet furnished 15.6 per cent of protein. These rats were the young of animals which received an abundance of fat-soluble A during the gestation and suckling period.

Ration III.		Minerals per 100 Gm. of Mixture.	
Soy beans.....	50	NaCl.....	0.921
Lard.....	20	K ₂ HPO ₄	2.48
Cornstarch.....	23	CaSO ₄ ·H ₂ O.....	0.016
Minerals.....	7	CaCl ₂	0.957
		Ca lactate.....	2.629

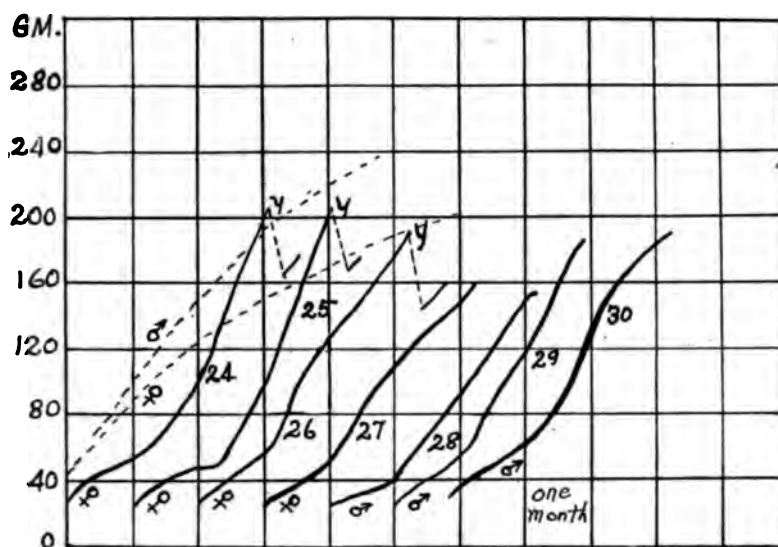


CHART V. Curves of growth of rats fed Ration II, consisting of soy beans, a suitable mineral mixture, and butter fat. This ration furnished 18.7 per cent of protein.

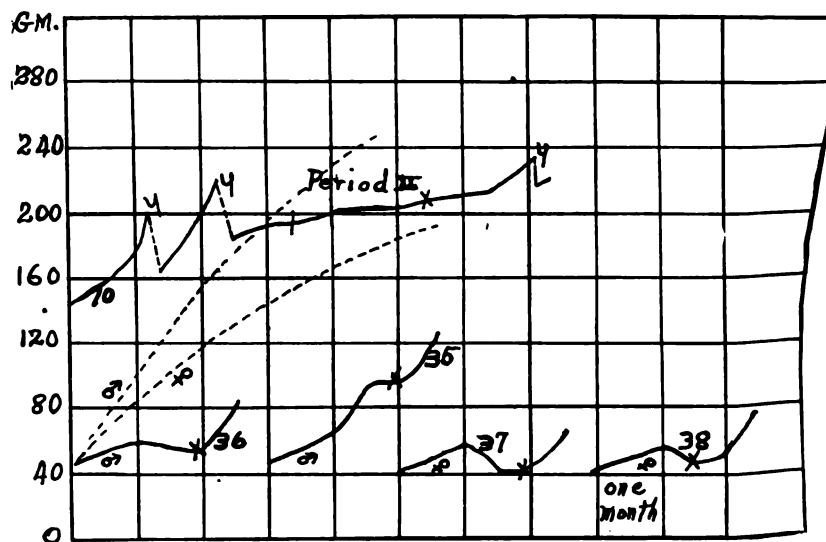


CHART VI. Immediately following the suckling period the young were placed on a diet (Ration IV) of purified foodstuffs in which the fat-soluble factor was lacking. The mineral content of the diet was similar to that of milk. The addition of butter fat (indicated by X) produced a marked improvement in the young. During Period II the adult animal received the purified ration.

Ration IV.

Casein.....	28
Lard.....	27
Cornstarch.....	32
Minerals as in milk.....	8
Agar.....	5
Water-soluble B from wheat embryo.	

ON THE INFLUENCE OF FOOD AND TEMPERATURE UPON THE DURATION OF LIFE.

BY JACQUES LOEB AND J. H. NORTHROP.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, September 4, 1917.)

I. Introductory Remarks.

In order to find out the nature of the causes which determine the natural duration of life of metazoa a quantitative method is required, which permits us to represent the duration of life as the numerical function of one variable. Starting from the idea that chemical conditions in the organism are one of the main variables in this case, one of us raised the question whether there was a definite temperature coefficient for the duration of life and whether this temperature coefficient was of the order of magnitude of that of a chemical reaction.¹ The first experiments were made on the unfertilized and fertilized eggs of the sea urchin and could only be carried out at the upper temperature limits of the organism, since at ordinary temperatures this organism lives for years. In the upper temperature region the temperature coefficient for the duration of life was very high, probably on account of the fact that at this upper zone of temperature death is determined by a change of the nature of a coagulation or some other destructive process. Moore,² at the suggestion of Loeb, investigated the temperature coefficient for the duration of life for the hydranth of a tubularian at the upper temperature limit and found that it was of the same order of magnitude as that previously found for the sea urchin egg. In order to prove that there is a temperature coefficient for the duration of life throughout the whole scale of temperatures at which an organism can live experiments were required on a form whose duration of life was short enough to

¹ Loeb, J., *Arch. ges. Physiol.*, 1908, cxxiv, 411.

² Moore, A. R., *Arch. Entwicklungsmechn. Organ.*, 1910, xxix, 287.

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measure the duration of life even at the lowest temperatures. Organisms especially fit for this purpose are insects.

We selected for this purpose the fruit fly (*Drosophila*) which can easily be raised in large numbers on a suitable culture medium in Erlenmeyer flasks. Since Metchnikoff pointed out that the poisons produced by bacteria in the intestine may shorten the duration of life it was necessary to work with flies whose whole body (the intestine included) was entirely free from microorganisms. We succeeded in producing such cultures of flies free from all microorganisms with the aid of a combination of methods introduced by Bogdanow³ and by Delcourt and Guyénot.⁴ Such flies will be designated as "aseptic." We have already published a preliminary report on some of our experiments⁵ and intend to give in this paper the full results of our investigations.

The results published on aseptic flies, *i.e.*, flies free from microorganisms, in the preliminary paper, were as follows.

1. With a supply of proper and adequate food the duration of the larval stage is an unequivocal function of the temperature at which the larvæ are raised, and the temperature coefficient is of the order of magnitude of that of a chemical reaction, *i.e.*, about 2 or more for a difference of 10°C. It increases at the lower and is less at the higher temperatures.

2. The duration of the pupal stage of the fly is also an unequivocal function of the temperature and the temperature coefficient is for each temperature practically identical with that for the larval stage.

3. The duration of life of the imago is, with proper food, also an unequivocal function of the temperature and the temperature coefficient for the duration of life is within the normal temperature limits approximately identical with that for the duration of the life of the larva and pupa.

From this approximate identity of the temperature coefficients for the three stages of the life of the fly we drew the conclusion that the limiting factor for the duration of each of the three

³ Bogdanow, A. E., *Arch. Physiol.*, 1908, Suppl., 173.

⁴ Delcourt, A., and Guyénot, E., *Bull. Sc. France et Belg.*, 1911, xlv, 249. Guyénot, *Recherches expérimentales sur la vie aseptique et le développement d'un organisme en fonction du milieu*, Thèse de Paris, 1917.

⁵ Loeb, J., and Northrop, J. H., *Proc. Nat. Acad. Sc.*, 1917, iii, 382.

stages is a process affected in the same way by the temperature. If it be true that this terminating factor for the larval or pupal stage is the production of a certain type of substance in sufficient quantity (as suggested by the influence of thyroid substance on the termination of the tadpole stage in the frog) or the removal of an inhibiting substance, it follows that a similar cause may be likely to exist for the termination of the last stage in life or for the duration of life.

In this paper we will discuss more fully the influence of the two main factors determining the duration of life, namely, food supply and temperature.

II. Influence of Different Kinds of Food on the Duration of Life of the Imago.

In some insects the imago takes up no food (as, *e.g.*, in the silk worm) but the duration of the life of the imago of the fruit fly depends on the nature of the food, though in an altogether different way from that of the larva. The growth of the insect takes place in the larval stage while neither the pupa nor the imago grows. It was found that while the larvæ cannot grow on "glucose-agar"⁶ unless yeast is added, the imago can live as well on "glucose-agar" alone as when yeast is added. This difference need not surprise us since the larva needs food containing all the building stones required for the synthesis of the compounds of its body while the imago, which does not grow, can live on food which is lacking in certain ("accessory"?) substances found in the yeast, presumably because such accessory substances are no longer needed in the fully grown organism or are needed in such small quantities that they can be supplied by the hydrolytic processes going on in its own cells.

Larvæ were raised on yeast at room temperature and the newly hatched flies were then put immediately after hatching upon dif-

⁶ "Glucose-agar," which proved an excellent culture medium for the flies in our experiments, had the following usual composition. 1 pound of beef was freed from fat, put into 1 liter of water, and placed in the refrigerator over night; boiled 30 minutes, filtered, 20 gm. of agar were added, and boiled till dissolved; 10 gm. of peptone added and boiled 20 minutes; neutralized to phenolphthalein, boiled 7 minutes, cooled to 60°; 2 eggs added, filtered, 20 gm. of glucose added.

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ferent culture media and the duration of life was noticed. All experiments were made with aseptic flies under aseptic conditions in thermostats at 25° and 30° respectively.

It was found (Table I) that on agar alone or on agar with the necessary salts the imagos lived less than 2 days at 25° while if dextrose and salts were added to the agar they lived over 8 days at 25°, and on "glucose-agar" they lived 28.5 days at the same temperature. As the experiment for 30° shows, they lived as long on "glucose-agar" alone as when yeast was added.

TABLE I.
Effect of Food on Duration of Life of the Imago (Both Sexes).

Food.	1 gm. washed agar + 100 cc. H ₂ O.	1 gm. agar 0.1 " K ₂ HPO ₄ 0.1 " MgSO ₄ 100 cc. H ₂ O.	1 gm. agar 0.1 " K ₂ HPO ₄ 0.1 " MgSO ₄ 2.0 " dextrose 100 cc. H ₂ O.	"Glucose-agar."		"Glucose-agar" + 5 gm. yeast per 100 cc.
				a.	b.	
Temperature ..	25°	25°	25°	25°	30°	30°
Average duration of life, days.....	1.92	1.75	8.25	28.5	13.7	13.1

TABLE II.
Effect of Sex (30°, "Glucose-Agar" Food).

Sex.	♂♂ ♀♀	♂♂	♀♀
Duration of life, days.....	13.1	15.7	13.3

In these experiments both sexes were used. It was found that the isolated males lived a little longer than the isolated females or the males when mixed with females (Table II). "Glucose-agar" served as food.

III. Influence of Temperature on the Duration of the Larval Period.

For these experiments the aseptic cultures were kept in water-jacketed incubators regulated to within $\pm 0.1^{\circ}\text{C}.$, and containing water so that the humidity was always about 100 per cent. The cultures of aseptic larvæ were made in 120 cc. Erlenmeyer flasks

containing 10 gm. of yeast, 15 cc. of water, and absorbent cotton. Slight variations in the amount of water, cotton, etc., do not affect the rate of growth. The flasks were sterilized in the autoclave for 30 minutes. Aseptic flies of both sexes were put in and allowed to remain 15 hours at room temperature, during which time a number of eggs were laid. The flies were then removed by the aseptic method devised by Delcourt and Guyénot and the flasks with the eggs put into an incubator. The larvæ hatch in a few hours after the eggs are laid, and at the time the flies were removed from the flask most of the larvæ had already hatched. The duration of the life of the larvæ was reckoned from the time the eggs were placed in the incubator to the time the pupæ were formed. Six to ten cultures were made for each temperature. The figures in Table III are the sums of the number of pupæ forming in the separate flasks on the day noted. In computing the averages for the time required to reach the pupal stage the middle of the interval at which the pupæ were formed was used; i.e., if 48 pupæ formed between the 4th and 5th day they were all considered to have taken 4.5 days to form.

We could not well use temperatures lower than 10° since the pupæ did not hatch at that temperature and it was obvious that the growth of the larva was no longer normal. Temperatures above 27.5° no longer accelerated growth.

If we compute from these values the temperature coefficient Q_{10} for the larval period (Table IV, Q_{10}) we find that it is of the order of magnitude of that of a chemical reaction, namely, 2 or more for a difference of 10°C. The temperature coefficients show, however, the irregularities characteristic of all the temperature coefficients for life phenomena, namely, increasing at the lower limit and diminishing at the higher limit. This peculiarity appears also in ordinary chemical reactions, but to a much less degree; but it appears more approximately to the same extent as in life phenomena in chemical reactions catalyzed by enzymes. It seems more natural to assume, as has been done by Arrhenius and others, that these deviations in the temperature coefficients are due to secondary effects of the temperature (e.g., upon viscosity or the state of aggregation of catalyzer or the injury to the catalyzer or some other variable) than to conclude that the temperature coefficient does not indicate a chemical (enzyme) reaction.

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It is well known that at the upper temperature limits the temperature coefficient of enzyme reactions falls off again when the

TABLE III.
Influence of Temperature on Duration of Larval Period of Drosophila.

Days elapsed after hatching of egg.		Number of pupæ formed at							
Day counted.	Average time.	10**	15**	20**	25°		27.5***	30.0**	31.5***
					a.*	b.**			
1-2	1.5								
2-3	2.5								
3-4	3.5					4	156	93	63
4-5	4.5				53	29	105	129	333
5-6	5.5				137	18	12	6	254
6-7	6.5			47	78				52
7-8	7.5			65	36				
8-9	8.5			68					
9-10	9.5			16					
13-14	13.5		2						
15-16	15.5		13						
17-18	17.5		27						
19-20	19.5		14						
21-22	21.5		6						
39-48	43.5	5							
49-58	53.5	20							
59-68	63.5	13							
69-78	73.5	3							
Total number of pupæ		41	62	196	304	51	273	228	702
Average duration of larval period in days (from egg to pupation).....		57.0	17.8	7.77	5.82	4.76	4.15	4.12	4.92

* Flies used were of the 20th to 22nd aseptic generation.

** Flies used were of the 29th to 31st aseptic generation.

temperature rises beyond a certain point. We were interested to know whether the same was true for the duration of the larval stage of the fruit fly, and found this to be the case (Table IV,

flies of the 30th to 32nd aseptic generation). On account of this probably secondary effect of the temperature the curve for the rate of larval development (the reciprocal value of the duration $\frac{100}{\text{time}}$) becomes from 10 to 30° (Fig. 1) almost a straight line; and from

TABLE IV.

Effect of Temperature on Rate of Growth of Aseptic Larvæ (Fed on Yeast).

Temperature.	Days required to reach the pupal stage.	Rate ($\frac{100}{\text{time}}$)	Q ₁₀
	Flies of the 20th to 22nd aseptic generation.		
°C.			
10	57 (pupæ do not emerge).	1.75	} 10.0 4.0 1.78 1.99
15	17.8	5.62	
20	7.77	12.85	
25	5.82	17.2	
30	4.12	24.25	
	Flies of the 30th to 32nd aseptic generation.		
25	4.76	21.0	} 1.74 -1.58
27.5	4.15	24.1	
31.5	4.92	20.3	

25 to 31.5° (Fig. 2) a curve concave on the lower side similar to that found for many simple enzyme reactions.

We noticed that the duration of the larval stage for the 20th to 22nd generation of aseptic flies was slightly longer than for the 30th aseptic generation and this was true for all temperatures and for all the stages. We cannot account for this difference at present.

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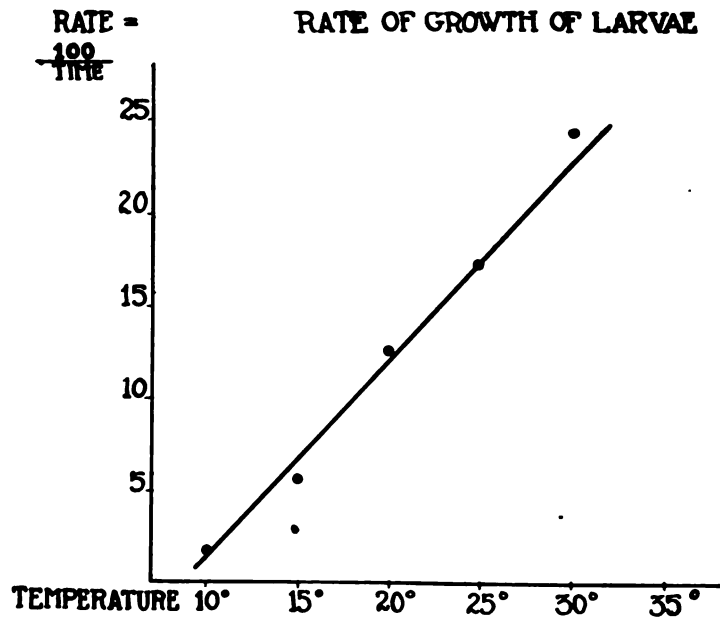


Fig. 1.

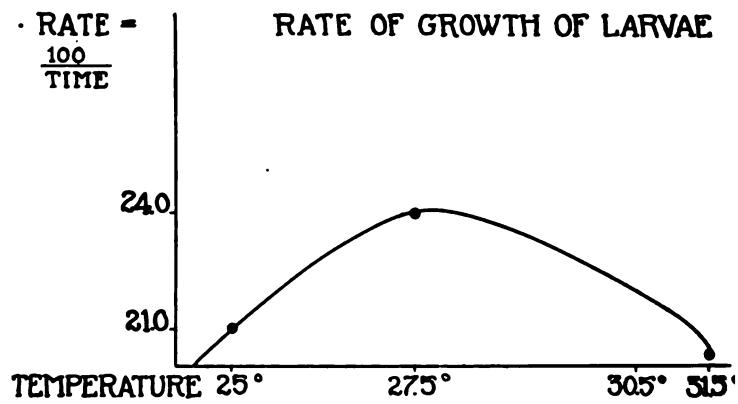


Fig. 2.

IV. Influence of Temperature on the Duration of the Pupal Stage.

Table V contains the data for determining the influence of temperature on the duration of the pupal stage. The stage terminates with the emergence of the imago from the cocoon. If we determine the time from the laying of the eggs to the emergence

TABLE V.
Egg-Imago and Pupal Period.

Number of days after larvæ emerge from egg.	Number of imagos which emerge at				
	15**	20**	25**	27.5***	30**
1.5					
2.5					
3.5					
4.5					
5.5					
6.5				58	30
7.5				205	114
8.5			6	17	39
9.5			151		
10.5			129		
11.5		4	23		
12.5		21			
13.5		36			
14.5		24			
15.5		21			
16.5		4			
17.5		5			
28.5	9				
30.5	25				
32.5	16				
34.5	10				
36.5	1				
Total number of imagos.	61	115	309	280	183
Number of days egg-imago...	31.5	14.10	10.05	7.35	7.55
Number of days egg-pupæ (Table III).....	17.8	7.77	5.82	4.15	4.12
Duration of pupa stage.....	13.7	6.33	4.23	3.20	3.43

* 20th to 22nd generation.

** 30th to 32nd generation.

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of the imago, and deduct from this the time from egg to beginning of pupation (Table III), we get the influence of temperature on the duration of the pupal stage. The temperature coefficient and the rate of development for the different temperatures are given in Table VI. In Fig. 3 the rate curve is plotted which is again between 15 and 30° approximately a straight line.

TABLE VI.
Temperature and Rate of Development of Pupæ. 20th to 22nd Aseptic Generation.

Temperature. °C.	Days as pupæ.	Rate ($\frac{100}{\text{time}}$)	Q ₁₀
15	13.7	7.2	5.0
20	6.33	15.8	
25	4.23	23.7	
30	3.43	29.15	

RATE OF DEVELOPMENT OF PUPAE

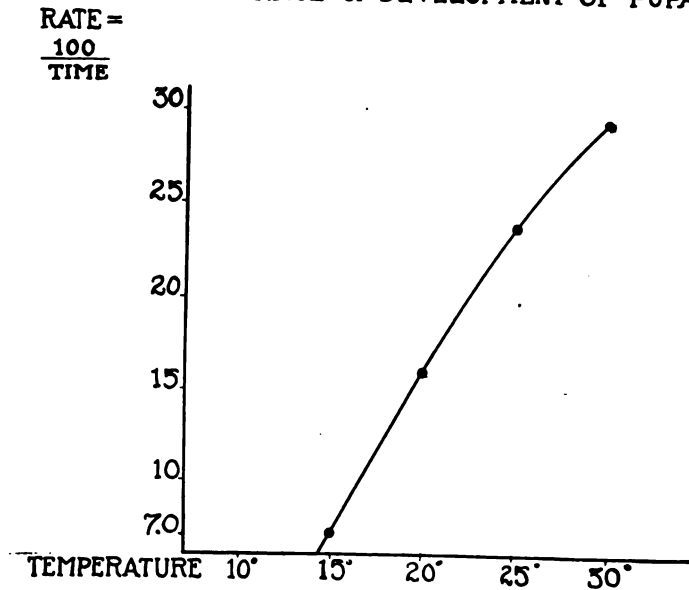


Fig. 3.

V. Influence of Temperature on the Duration of Life of the Imago.

Aseptic larvæ were raised at room temperature (18–20°) on yeast and the flies were removed aseptically from the culture flasks within 15 hours of the time they emerged. They were

TABLE VII.
Duration of Life of Imago on "Glucose-Agar."

10°		15°		20°		25°		30°	
Days.	No. of dead.	Days.	No. of dead.	Days.	No. of dead.	Days.	No. of dead.	Days.	No. of dead.
17.5	1		1	14.5	1	12.5	6	3	31
24.5	2		4	24.5	5	22.5	30	8	54
31.5	1		0	34.5	14	32.5	20	13	66
52.5	1	38.5	1	44.5	23	42.5	14	18	20
59.5	5	45.5	2	54.5	6			23	43
66.5	1	52.5	3					28	14
73.5	3	59.5	3						
80.5	0	66.5	12						
87.5	2	73.5	18						
94.5	2	80.5	20						
101.5	5	87.5	12						
108.5	2	94.5	11						
115.5	5	101.5	3						
122.5	17	108.5	8						
129.5	14	115.5	4						
136.5	12	122.5	27						
143.5	19	129.5	7						
150.5	7	136.5	7						
157.5	5								
164.5	1								
Total number of flies..	105		143		49		70		228
Average duration of life, days...	120.5		92.4		40.2		28.5		13.6

placed into sterile 500 cc. Erlenmeyer flasks containing 25 cc. "glucose-agar." This food is, as stated, perfectly adequate for the flies but is inadequate for the larvæ which cannot develop on it into flies. This was an important point in the method of our experiments. In order to determine the duration of the life of

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the imago for a given temperature a definite number of flies were put into a flask, and the number of those which died was ascertained for each day. These flies laid eggs. If the larvæ hatching from these eggs had been able to develop into new flies it would have become impossible to find out the exact death rate of the old flies, since constantly new flies would have been added. This source of error was avoided by using "glucose-agar" as a culture medium for the old flies on which the eggs laid by the flies during

TABLE VIII.
Duration of Life of Imago on "Glucose-Agar."

27.5°		31.5°		33.1°		35°		37.4°	
Days.	No. of dead.	Days.	No. of dead.	Days.	No. of dead.	Days.	No. of dead.	Minutes.	No. dead per 100.
2	10	3	21	1.5	48	0.5	61	10	9
4	13	5	22	2.5	40	1.16	27	30	37
6	14	7	43	3.5	17	1.66	27	50	25
8	10	9	40	4.5	9	2.16	2	70	26
10	14	11	7	5.5	1			90	4
12	9								
14	15								
16	28								
18	6								
20	1								
22	3								
24	3								
Total number of flies..	126		133		115		117		101
Average duration of life, days...	11.1		6.87		2.41		0.95		0.032

the experiment could not reach the imago stage. By special tests (smears and cultures from dead flies) it was ascertained that all the cultures used in the experiments remained sterile to the end of the experiment. The flasks containing the flies were plugged with cotton. Sufficient food and oxygen were present since cultures containing 30 to 40 flies had the same average duration of life as those containing 10 to 15.

Five to ten separate cultures were used at each temperature, each containing 5 to 20 flies. Several cultures with relatively few

flies in each were used since it was more convenient to count the number of dead flies under these conditions. The time was reckoned in the same way as for the larval and pupal periods.

Table VII gives the statistical results of the duration of life of the imago on "glucose-agar." The flies used were of the 20th to

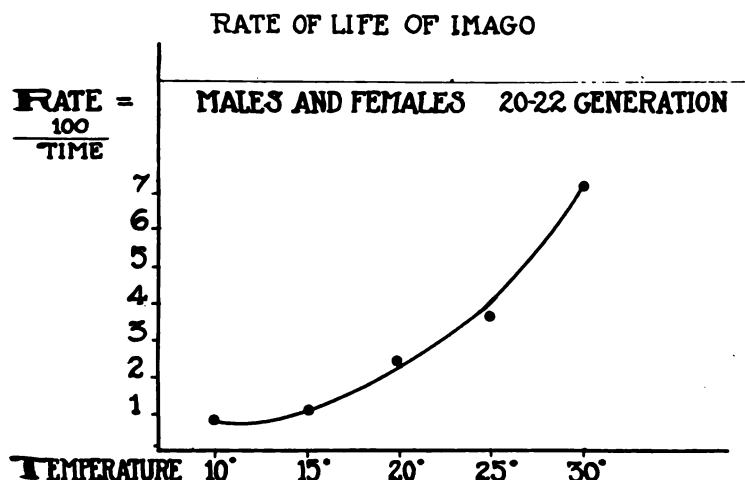


Fig. 4.

22nd aseptic generation. Table VIII gives the duration for the upper limit of temperatures, 27.5–37.4°. Both sexes were used indiscriminately in these experiments. In Table IX the average results are tabulated and the temperature coefficients for 10° are given. A temperature of 10° or lower is harmful for the organism, as are temperatures above 30°. In Table IX the temperature coefficients of the duration of life of the imago are computed and the reciprocal value of the duration of life—the rate at which an animal "gets through" with life—is calculated. If we plot this curve (Fig. 4) which corresponds to the rate curve of the larval and pupal stage we find that in the case of the imago it is no longer a straight line but more what we should expect for a chemical reaction curve. The reason that Curve 4 corresponds more to a chemical reaction curve than Curves 1 and 3 is that the rate does not decrease at the higher temperature limits. This decrease is then a merely secondary phenomenon but it is responsi-

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ble for transforming the rate curve into a straight line; all of which corroborates our previous statement that the straight line character of the curve does not militate against the assumption that we are dealing in all three curves with a temperature coefficient of the order of that of a chemical reaction.

TABLE IX.
Temperature Coefficient of Duration of Life of Imagos. Males and Females Fed on "Glucose-Agar."

Temperature.	Duration of life of imago.	Rate ($\frac{100}{\text{time}}$)	Q ₁₀
	20th to 22nd generation.		
°C.	days		
10	120.5	0.83	} 1.70 5.25 1.99 4.4
15	92.4	1.08	
20	40.2	2.49	
25	28.5	3.51	
30	13.6	7.35	
	30th to 32nd generation.		
27.5	11.1	9.00	} 3.3 630 137 10 ⁴
31.5	6.87	14.55	
33.1	2.41	41.50	
35	0.95	105.2	
37.5	0.032	3,125.0	

In conclusion we will give in Table X the total duration of life and its temperature coefficients for the temperatures at which the animal can complete its cycle.

VI. The Mortality Curve.

When we plot the number of flies which die during successive days in terms of percentage of the original number of flies we get

TABLE X.
Total Duration of Life.

Temperature.	Total duration of life.	Rate ($\frac{100}{\text{time}}$)	Q ₁₀
°C.	days		
15	123.9	0.81	} 5.0 3.0 3.0
20	54.3	1.84	
25	38.5	2.67	
30	21.15	4.65	

that curve of the death rate usually given in life insurance statistics, namely, a probability curve, the ascending branch of which is a little steeper than the descending branch. The death rate of a population of aseptic male flies on "glucose-agar" at 30° is thus given in Fig. 5 and Table XI.

Miss Chick⁷ has stated that bacteria are killed by disinfectants at a rate corresponding to that of a monomolecular chemical reaction, *i.e.*, that in each interval of time the same percentage of individuals alive at this time is killed. She was probably led to such an assumption by the fact that the ascending branch of the mortality curve in her experiments was generally very steep. The agencies used by her for killing the bacteria were so powerful

TABLE XI.
Rate of Death at 30°. Males.

Time.	No. of dead.	No. of dead in interval.	Percentage of original number dying in interval.
days			
3.5	5	5	4.8
7.5	19	14	13.5
13.0	60	41	39.4
18	78	18	17.2
23	93	15	14.4
28	104	11	10.6

⁷ Chick, H., *J. Hyg.*, 1910, x, 237.

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that the ascending branch became almost a vertical line, thus escaping detection. Hence she noticed usually only the less steep descending branch which could be interpreted as a monomolecular curve for the reason that her experiments lasted only a short time. In Fig. 6 we give the frequency curve of deaths of a culture of males for a very high temperature, namely, 39.45° . The ascending branch of the curve is steeper than that for the lower

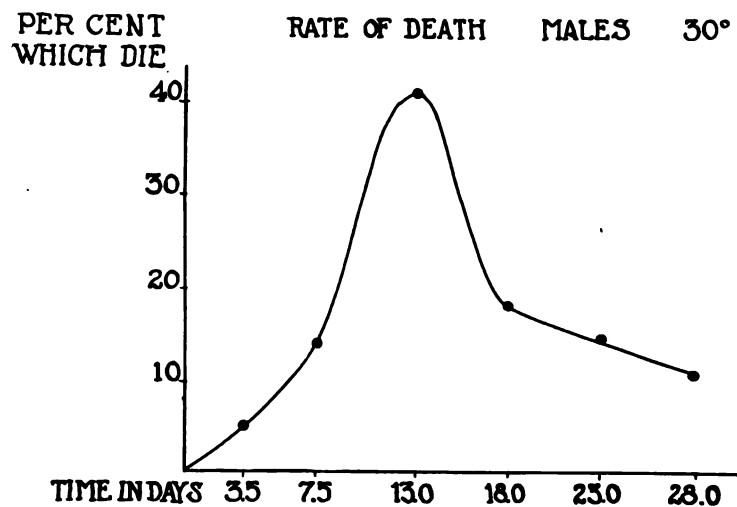


Fig. 5.

temperature in Fig. 5, but the fact that we are dealing with a probability curve is still very clear. Table XII gives the observations on which this curve is based.

The fact that the frequency curve of deaths is that of a probability curve shows that the difference in the duration of life of different individuals for the same temperature is due to individual variation. Incidentally it may be stated that observations on the rate of death of *Fundulus* embryos under the influence of acids, alkalis, and potassium salts show that the mortality curve in these cases is also a probability curve, the descending branch of which is less steep than the ascending branch. This difference may possibly be ascribed to a slight adaptational effect of the destructive agency.

30 to 40 flies were put in test-tubes and placed in a water bath kept at $39.45^\circ \pm 0.02^\circ$, then taken out at time stated and left at room temperature over night. The number of live and dead males was counted after 14 hours.

TABLE XII.
Rate of Death at 39.45° . Males.

Time. <i>min.</i>	No. of dead.	Total per cent dead.	Per cent dying in interval.
25	30	4.5	4.5
30	130	17.0	12.5
35	177	34.6	17.6
40	318	54.6	20.0
45	489	72.0	17.4
50	550	82.9	10.9
55	440	88.2	5.3
60	364	91.2	3.0
65	532	97.5	(5.3)
70	350	98.9	1.4
75	609	99.7	0.8

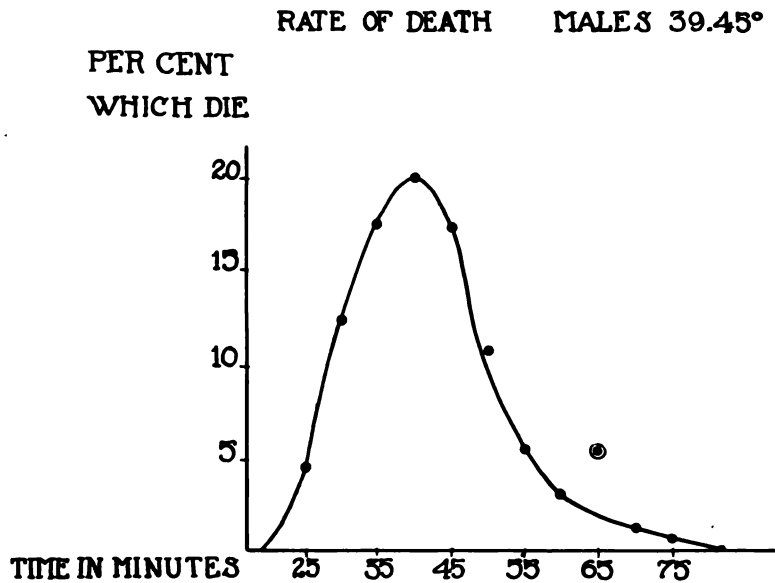


Fig. 6.

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VII. Comparison of the Temperature Coefficients for the Larval and Pupal Stage with That for the Duration of Life.

The life of *Drosophila* is normal only within temperatures above 10° and below 30° or roughly between 15° and 25°C. At 10° the larvæ reach the pupal stage but the animals then die without ever emerging from the cocoon. The imago, however, lives at 10° C. At 5° or less the duration of life of the imago is less than a week while at 15° it is 92.4 days. The fact that at 5° the duration of life is less than at 15°C. shows that the former temperature is incompatible with the life of the organism. At temperatures between 27.5° and 31.5°, at which temperature the coefficient for the rate of growth of the larvæ becomes negative, life is also no longer normal. The life of the fly is normal between 15° and

TABLE XIII.
Temperature Coefficients of Various Stages of Development.

Temperature. °C.	Q ₁₀ for rate of		
	Larvæ.	Pupæ.	Imagæ.
15-20	4.0	5.0	5.25
20-25	1.78	2.24	1.99

25°C., and it is, therefore, for this range that a comparison of the temperature coefficients for the three stages becomes permissible. Table XIII shows the temperature coefficients for 15-20° and 20-25° and it is obvious that they are approximately the same for all three stages.

As we have already stated in a previous paper, this proximity of the three values suggests a proximity of the cause limiting the three stages. If the limiting factor for the larval and pupal stages be the production or destruction of a substance ("hormone") the same limiting factor may be suspected for the duration of life. Experiments made by Northrop⁸ show that thyroid has no influence on metamorphosis in the fly.

⁸ Northrop, J. H., *J. Biol. Chem.*, 1917, xxx, 181.

SUMMARY.

1. The paper proves the existence of a definite temperature coefficient for the duration of life of the fruit fly (*Drosophila*).

2. Since the experiments were made with fruit flies free from microorganisms death cannot be ascribed to bacterial poisoning.

3. The temperature coefficient for the duration of life of the fruit fly is approximately identical with the temperature coefficients for the duration of the larval and pupal stage between 15° and 25°C., i.e., within the limits where development is normal.

4. The duration of the three stages in the life of aseptic *Drosophila*, and the total duration of life is, for temperatures between 10° and 30°, as follows.

Temperature.	Duration (in days) of			
	Larval stage.	Pupal stage.	Life of imago.	Total duration of life from egg to death.
°C.				
10	57	Pupæ die.	120.5	177.5 + X
15	17.8	13.7	92.4	123.9
20	7.77	6.33	40.2	54.3
25	5.82	4.23	28.5	38.5
27.5	(4.15)	3.20		
30	4.12	3.43	13.6	21.15

5. Small variations in the duration of life were noticed in different aseptic generations of the flies; in the 32nd generation the rates were all slightly quicker than in the 20th to 22nd generation.

6. Aside from the temperature the nature of food influences the duration of life and an "adequate" food supply is presupposed for work on the influence of temperature as stated in the previous paragraphs. An adequate food supply for the growing larva includes yeast, while for the adult fly which no longer grows "glucose-agar" (with or without yeast) is sufficient.

7. The observations on the temperature coefficient for the duration of life suggest that this duration is determined by the production of a substance leading to old age and natural death or by the destruction of a substance or substances which normally prevent old age and natural death.

THE EFFECT OF PROLONGATION OF THE PERIOD OF GROWTH ON THE TOTAL DURATION OF LIFE.

By JOHN H. NORTHROP.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, September 4, 1917.)

It is a well known fact that the growth of an organism may be more or less retarded by supplying it with food insufficient either in quantity or quality. It has been shown¹ that in the case of "aseptic" *Drosophila* the growth of the larvæ may be greatly retarded by growing them on sterile fruit or other substances containing no yeast. The deficiency in this case is probably of the nature of the "vitamines" or growth-promoting substances which have been so thoroughly investigated by Funk, McCollum, Osborne and Mendel, and others. It has also been shown¹ that this prolongation of the larval period is without effect on the duration of the pupal period. It seemed of interest to determine whether the duration of the next stage—the imago—was also independent of the time required to reach that stage. The present experiments were undertaken in order to determine this point. Indirect evidence on this point has been brought forward by Osborne, Mendel, and Ferry,² who have shown that the menopause in female rats is delayed by stunting. Unfortunately their animals died from infection so that the normal duration of life was not determined.

In all these experiments the cultures of flies were, as stated, completely free from microorganisms and the experiments were made in sterile culture media. The Erlenmeyer flasks containing the flies were previously sterilized in the autoclave for 30 minutes as was also the banana or yeast used. The transferring of the sterile flies into a new flask or their removal from the flask was done by the device given by Delcourt and

¹ Northrop, J. H., *J. Biol. Chem.*, 1917, xxx, 181.

² Osborne, T. B., Mendel, L. B., and Ferry, E. L., *Science*, 1917, xlv, 294.

Guyénot,³ which excludes contamination of the fly by micro-organisms.

In the experiments described in Table I the eggs were laid on 10 gm. of sterile banana in 120 cc. Erlenmeyer flasks. The parent flies were left in the flask for 4 days, during which time they laid a considerable number of eggs, and were then removed. After some time, as noted in the table, a sterile suspension of yeast in water was added. Upon emerging from the pupal stage the flies hatched from these eggs were transferred aseptically to 500 cc. flasks containing glucose-agar, on which they live as long as on yeast.⁴ The duration of life was then noted. Each experiment is the combined result of from six to nine different cultures. The flies used were of the thirtieth aseptic generation.

The experiment was made in an incubator at 27.5°C.

Table I shows the following facts. When yeast is added at the beginning of the experiment the average total duration of life is 19.3 days at 27.5°, while when the yeast is added on the 8th day of the larval period the total duration of life is 25.5 days at 27.5°, and if the yeast is added on the 10th day the average total duration of life of the fly is 28.9 days. This increase in the duration of life is wholly due to the lengthening of the egg-larval-pupal period by inadequate food supply to the early stages, since the average duration of life of the imago is, as the table shows, approximately the same, namely, between 10.5 and 11.9 days. The larval period was, therefore, prolonged from 8 to 17 days, i.e., over 100 per cent. When the yeast was added on the 12th day or later the life of the larvæ could no longer be saved and they died in the larval stage without being able to complete their development. It must be remembered that the absolute figures given in Table I apply only to the temperature of 27.5°C., and that they would have to be altered for other temperatures.

³ Delcourt, A., and Guyénot, E., *Bull. Sc. France et Belg.*, 1911, xlv, 249.

⁴ Loeb, J., and Northrop, J. H., *J. Biol. Chem.*, 1917, xxxii, 103.

TABLE I.

Effect of Prolongation of Larval Period on Total Duration of Life.
Temperature 27.5°C.

Number of days after removal of flies.	Experiment 1.	Experiment 2.	Experiment 3.	Experiment 4.	Experiment 5.
0	1-2 mm. larvæ. Yeast added.	1-2 mm. larvæ.	1-2 mm. larvæ.	1-2 mm. larvæ.	1-2 mm. larvæ.
4	Pupæ form.*	Yeast added.			
8	Flies emerge.	Pupæ form.	2-3 mm. larvæ. Yeast added.		
	Number of flies which die.				
10	10			2-3 mm. larvæ. Yeast added.	
11			Pupæ form.		
12	13	Flies emerge.			Larvæ dead.
13		Number of flies which die.		Pupæ form.	
14	14				
15		5	Flies emerge.		
16	10		Number of flies which die.		
17		22		Flies emerge.	
18	14			Number of flies which die.	
19		35	7		
20	9				
21		44	33		
22	15				
23		33	32	5	
24.5	28		41	2	
25		61			
26	6				
27		50	24	15	
28	1				
29	3	21	19	7	

TABLE I—*Concluded.*

Number of days after removal of flies.	Experiment 1.	Experiment 2.	Experiment 3.	Experiment 4.	Experiment 5.
31		6	21	5	
33	3	6	11	8	
36			2	2	
38	.			1	
Total number of flies.....	126	283	190	45	
Average total duration of life.....	19.3	23.5	25.5	28.9	
Egg-larval-pu- pal period...	8.0	12.0	15.0	17.0	
Duration of life of imago.	11.3	11.5	10.5	11.9	

* All pupæ formed within a period of 3 days. The 2nd day of pupation is taken as the average time and is the one given in the table. The same approximation is made for the duration of the pupal period. The exact time could not be ascertained in this case since it was impossible to count the imagos as they emerged, owing to the necessity of keeping them sterile.

SUMMARY.

The experiments show that if the larval period is prolonged by inadequate feeding the total duration of life can be prolonged. This simply shows that the relative duration of each of the three stages is independent of that of the other two stages; and this would harmonize with the idea that the duration of each of the three stages is determined by the formation (or by the disappearance) of a definite specific substance, as stated in a previous paper.⁵

⁵ Loeb and Northrop, *Proc. Nat. Acad. Sc.*, 1917, iii, 382

THE EXISTENCE OF A GASTRIC LIPASE.*

By MARY HULL AND ROBERT W. KEETON.

(*From the Laboratories of Physiological Chemistry and Pharmacology of the Northwestern University Medical School, Chicago.*)

(Received for publication, August 3, 1917.)

The existence of a gastric lipase has been a much debated subject since Volhard (1) published his series of three articles in 1900 and 1901. The literature up to these dates was thoroughly revised. Investigations, stimulated by these articles, extended over a period of 12 years and have been summarized by Davidsohn (2) with a complete bibliography.

By means of test meals, Volhard (1), Stade (3), Zinsser (4), Meyer (5), Sedgwick (6), and Heinsheimer (7) have shown unquestionably that lipolytic activity for emulsified fats is exhibited by the stomach. However, they are not agreed as to the concentration or the source of the enzyme, whether it be a gastric or pancreatic product.

Attempts to recover the lipase from pure juice (Volhard, 1, Pekelharing, 8, Heinsheimer, 7, Laqueur 9) or to exclude a reflux from the intestines (London, 10, Meyer, 11, Mueller, 12) have resulted in a fat-splitting power which is much below that found from test meals.

Extracts of mucosa (Fromme, 13, Sedgwick, 6, Van Herwerden, 14) gave a decided lipolytic activity which Inouye (15) and Meyer (5, 11) were unable to confirm. Davidsohn (2), in studying the properties of gastric lipase—reaction to acid, alkalies, and sodium fluoride—has adduced striking experimental evidence for its existence. However, the failure of Volhard and his workers to rule out a reflux of the pancreatic juice from the intestines, and the low values reported by every one who has worked with even an approximation of pure juice throw some doubt on its existence in appreciable concentrations, if not on its existence at all. For these reasons it appeared desirable to attack the problem again, with the idea of studying the pure juice.

Methods.

The experiments were conducted upon dogs, some with gastric fistulas, others with Pavlov accessory stomachs, and still others

* The data included were presented by Miss Hull in partial fulfillment of the requirement for Master's degree from Northwestern University, June, 1917.

in which the pylorus had been blocked. The dogs with the Pavlov stomachs were the same as those employed in the investigation of Professor Long on the persistence of trypsin in contact with gastric juice (16). One other dog having a Pavlov stomach and his splanchnic nerves cut on both sides was operated upon for this investigation and has continued in most excellent condition for subsequent work. The pylorus was blocked in two ways. In the first series, the abdomen was opened under local anesthesia and a ligature slipped about it. In one other case a complete separation was made under ether with the establishment of a duodenal fistula for furnishing fluids to the animal. Animals in this group were killed after a short period.

In order to secure a larger secretion of gastric fluids some stimulus of the stomach was generally necessary. This stimulus was accomplished in many instances through injections of gastrin and other gastrin bodies which were being studied by one of us in conjunction with Drs. F. C. Koch and A. B. Luckhardt of the University of Chicago. The injections were made into the muscles of the lumbar regions.

Estimation of the Lipase.

In most cases the extent of lipolytic activity was measured by determining the amount of a fatty oil or simple ester split in the incubation of the substance in contact with the gastric juice. The estimations were made upon both ethyl butyrate and neutral olive oil as substrates. Experience soon showed that it made little difference which was used. 1 to 1.5 cc. of gastric juice were added to 25 cc. of a saturated water solution of neutral ethyl butyrate, incubated 2 hours, and then titrated (phenolphthalein as indicator) with 0.05 N NaOH, after the addition of 25 cc. of acid-free alcohol and 5 cc. of ether. The results are calculated to the amount of alkali that would be required to neutralize fatty acids split off by 100 cc. of the juice under similar conditions. Parallel blank tests which were run with boiled juices always gave a low titration value of 0.5 to 1.5 cc. As will appear below, there were many cases where the titration results were negative, indicating no enzymic action; but, when positive, the acid measured in this titration of ethyl buty-

rate, or corresponding titrations when olive oil was employed as the substrate, is actually acid separated from the ester and not formed by some possible autolytic process taking place in the juice during the period of incubation. It is conceivable that organic acids with an appreciable titration value might be formed in this way and this possibility had to be excluded. It was found, in the first place, that the acids from the digests could be readily shaken out with ether, while no measurable acid came from the juice itself when treated in the same way. Secondly, a boiled juice, when incubated alone and under conditions which would favor autolysis, furnished no appreciable amount of acid on titration. These observations, coupled with those made on the boiled juice just referred to, show beyond question that we are dealing with acid bodies found through the action of the gastric juice secured from the fistula or Pavlov stomach on the substrate and not with autolytic products.

The method is, therefore, satisfactory for use. Some observations made with the Volhard method and one of its modifications will be referred to later for comparison. The fraction of gastric juices secured for examination always possessed some slight degree of acidity, mostly due to hydrochloric acid. This and the total acidity were found by the usual titration with methyl orange and phenolphthalein.

EXPERIMENTAL.

Variations in Diet.—In beginning observations on dogs with the accessory stomach, it soon became evident (as observed by so many previous workers in the field) that the titration results were often negative, but there was always a possibility that some factor had been left uncontrolled in the observation. The question of adaptation of diet presented itself quite naturally. To test this, juices were collected under varying types of diet as shown in the footnote to Table I. Reference to this table, however, shows that in all types of diet tested the percentage of negative experiments was high, and that, of the positive experiments, the quantity of fatty acid formed was quite small. It is evident, therefore, that some other explanation must be sought for the inconstant presence and low values of the lipase as found in these pure juices.

TABLE I.

Character of diet.*	Acid, per cent HCl.		Lipase, 0.05 N NaOH per 100 cc. of juice.		No. positive.	No. negative.
	Free.	Total.	Olive oil.	Ethyl butyrate.		
			cc.	cc.		
Mixed.....	0.30	0.34	4.9	4.7	5	2
Raw meat.....	0.35	0.39	4.5	4.5	3	2
Cooked meat.....	0.48	0.52	5.5	5.5	4	6
Fat meal, not emulsified.....	0.34	0.38	16.5	16.5	2	3
Fat meal, emulsified.....	0.22	0.25	7.7	7.7	4	2
Fat meal, emulsified plus gastrin injections.....	0.27	0.31	9.5	7.25	5	2

* Mixed diet: boiled beef, bread, and milk.

Raw meat: ground raw hamburger meat.

Cooked meat: ground boiled beefsteak.

Fat meal, not emulsified: boiled fat meat, ground and mixed with lard and butter.

Fat meal, emulsified: milk, raw egg, beaten up with olive oil.

Fasting Juice.—In the course of a piece of work published by one of us (17) attention was called to the high concentration of pepsin found in the juice from the stomach of the fasting dog. An examination of such a juice collected from an animal fed the day previous, secreted at the rate of 1.8 cc. per hour, no free acid, total 0.04 per cent, gave an activity of 80 cc. of 0.05 N NaOH measured by olive oil and 90 cc. measured by ethyl butyrate. The pepsin concentration was high. This experiment was very suggestive and was repeated a number of times, giving always this high figure, so that it came to be used as a method of obtaining a stock solution of the active juice for study. It appeared evident that the low acidity of the fasting juice might, after all, be the important factor, and this was tested by many experiments. Some observations bearing on the behavior of the ferment in presence of acid and alkali were therefore suggested.

Effects of Acids and Alkalies on the Lipase.—Our aim at this point was not to study exhaustively the properties of the enzyme, but rather to define the limits of its destruction so that we might be guided by these in searching for it.

Experiment 1.—The fasting juice was incubated (40°C.) with 4 cc. of a solution from an actively secreting stomach (free acid 0.51 per cent, total acid 3 per cent) for varying periods and then the activities of all the enzymes were estimated on ethyl butyrate as substrate with the following

Incubation period.	Lipolytic activity, 0.05 N NaOH.
	cc.
fasting juice (control).....	76.0
1 hr.....	4.0
2 hrs.....	5.0
3 ".....	4.0
18 ".....	4.0

This experiment was typical of several and showed the effect of a gastric solution, from an actively secreting stomach exhibiting itself no lipolytic activity, upon the gastric juice from the fasting stomach. When the solution from the actively secreting stomach was neutralized before exposure to the fasting juice to it the results are quite different, as seen in Experiment 2.

Experiment 2.—The above experiment was repeated with the neutralized solution of the acid in the juice from the actively secreting stomach before it was added to the fasting juice.

Incubation period.	Lipolytic activity, 0.05 N NaOH.
	cc.
fasting juice (control).....	80.0
1 hr.....	70.0
1 ".....	65.0
2 hrs.....	25.0
3 ".....	10.0

Experiments 3, 4, and 5 show the effect of acid and alkali and water on the lipolytic value of the fasting juice.

Experiment 3.—Incubation with HCl of the same titration value (0.5 N) as the actively secreted juice.

Incubation period.	Lipolytic activity, 0.06 N NaOH.
	cc.
Fasting juice (control).....	75.0
Mixture. 5 min.....	8.0
10 "	5.0
15 "	2.0
30 "	0.0

Experiment 4.—Incubation period 30 minutes with varying concentrations of acid.

Acid concentrations.	Lipolytic activity, 0.06 N NaOH.
	cc.
Fasting juice (control).....	90.0
Mixture. Water.....	75.0
0.10 per cent HCl.....	48.0
0.15 " " "	20.0
0.20 " " "	5.0
0.25 " " "	0.0

Experiment 5.—Incubation period 30 minutes with varying concentrations of alkali.

Alkali concentrations.	Lipolytic activity, 0.06 N NaOH.
	cc.
Fasting juice (control).....	75.0
Mixture. Water.....	65.0
0.10 per cent NaOH.....	8.0
0.15 " " "	5.0
0.20 " " "	0.0

We may conclude from these data that the gastric lipase is a very unstable body since activity is lost even in water solution and quite rapidly in solutions with increasing quantities of H and OH ions.

Recovery of the Lipase from a Stomach in Active Secretion.—Having defined roughly the limits as to acid concentration and the time of exposure to these within which one might still hope to secure a juice with lipolytic activity, we endeavored to attack more directly the explanation for the low activity of a juice

coming from an actively secreting stomach. Three possibilities present themselves for consideration. The origin of the lipase may be from a source analogous to mucus, being favored by periods of low secretory activity, and lost in the great dilution coming with the full secretion. It might obey the same laws as the other gastric enzymes, and yet on account of its sensitiveness to the H ions be destroyed before it could be estimated. Finally, we may be dealing with an intracellular enzyme which finds its way into the gastric juice quite by accident, or a product separated from the blood stream, and in this case its significance would be that of an excretion rather than a secretion.

Obviously the next experimental step was to neutralize the juice as soon as it could be collected. The Pavlov animal with splanchnics cut on both sides secreted, on a meal of meat and extractives, 25 to 30 cc. an hour (free acid 0.5 to 0.6 per cent). This juice was neutralized with NaOH as it dropped into the collecting container. Certainly not over 5 minutes could have elapsed from the moment of secretion until that of neutralization, and yet in no case could lipolytic activity be recovered; similar experiments on another animal gave no activity with a juice of 0.35 per cent free acid, but a small lipase content (2 cc. of 0.05 N NaOH) when the secretion level dropped to 0.22 per cent.

Table II shows the effect of neutralizing the juice (immediately and after 30 minutes) obtained from a stomach which has had its secretory activity depressed, but which is nevertheless exhibiting activity.

In order to get at the possible physiological rôle of the lipase it seemed worth while attempting to reduce the acidity within the stomach by the introduction of peptone. So the pylorus was blocked by a ligature in the manner previously described and the experiment was performed the next day (Table III).

One could hardly hope for a more perfect demonstration of the fact that any procedure which reduces the acidity, whether it be peptone injection or immediate neutralization with alkali, gives a high lipolytic activity.

With this explanation before us it is possible to interpret a large number of lipase estimations made in the early part of the work, which at first seemed to have no value. These had been made upon juice collected from gastric fistula dogs under stimu-

lation from gastrin bodies. Twenty-five experiments in all were run, simultaneous estimations of trypsin being made to check the regurgitation from the intestines. Table IV gives the four experiments from these twenty-five which showed lipolytic activity. In the others it was absent entirely or too low to be of much significance.

It will be noted that in only one of these cases was the acid present in appreciable concentrations. It is further of interest to note that in the two cases (Vb and VIb) in which there was re-

TABLE II.

Pavlov Stomachs.

Fat Meal: 50 Gm. Cooked Meat (Extractives Removed); 75 Gm. Lard; 25 Gm.

Crackers.

Animal.	Time of collection.	Volume.	Acid, per cent HCl.		Lipase, 0.05 N NaOH per 100 cc. of juice.	Remarks.
			Free.	Total.		
I	1½ hrs.	9.3	0.15	0.18	60.0	Juice neutralized after 30 min. intervals.
II	1½ "	17.3	0.18	0.19	75.0	"

Meal of meat and extractives. Collections of juice 12 hours after feeding.

I	30 min.	5.0	0.13	0.22	19.9	Neutralized at end of 30 min.
	30 "	3.75	0.22	0.26	16.6	"
	30 "	3.50			80.0	Neutralized as collected.
II	25 "	7.50	0.35	0.42	3.0	Neutralized at end of 25 min.
	25 "	4.0	0.27	0.33	13.0	"
	25 "	3.2			72.0	Neutralized as collected.

gurgitation of intestinal contents as shown by the trypsin present, there was no increase in the lipases.

In the earlier part of the work the pylorus was blocked in three animals and they were then injected with gastrin. In the majority of the cases a small lipolytic activity, corresponding to 4 to 10 cc. of 0.05 N NaOH, was obtained without any precautions being taken to control the acidity of the contents. Interpretation of the work at that time favored the view of the relative unimportance of the enzyme.

TABLE III.

Neutralization with Alkali and Peptone. Pylorus Blocked. Gastric Fistula.

Time.	Manipulation.	Vol- ume.	Acid, per cent HCl.		Lipase, 0.05 N NaOH per 100 cc. of juice.
			Free.	Total.	
9.00	Emptied stomach.	cc. 60.0	0.0	0.16	cc. 84
Intravenous injection of salt solution 150 cc.					
9.30	Collection. Neutralized after 30 min.	22.5	0.06	0.23	86.08
10.00	"	20.0	0.21	0.30	7.0
10.30	Collection. Neutralized as secreted.	9.0			78.3
11.00	Collection. Neutralized after 30 min.	12.0	0.07	0.23	79.0
11.30	"	10.0	0.07	0.23	63.0
12.45 65 cc. of 5 per cent peptone solution introduced into stomach. 1 cc. of gastrin injected into back muscles.					
1.15	Stomach emptied.	33.0	0.0	0.50	82.0
1.40	"	15.0	0.31	0.40	8.0
1.40 20 cc. of peptone solution introduced into stomach.					
2.05	Stomach emptied.	33.0	0.14	0.51	75.0
2.40	Stomach drained through fistula and neutralized after 30 min.	23.0	0.31	0.38	24.0

TABLE IV.

*Gastric Fistula Dogs.**Spontaneous Secretion and Secretion Stimulated by Gastrin Bodies.*

Animal.	Quantity.	Acid, per cent HCl.		Lipase, 0.05 N NaOH per 100 cc. of juice.	Trypsin, 0.2 N NaOH for amino-acid per 100 cc. of juice.
		Free.	Total.		
	cc.			cc.	cc.
III	4.5	0.04	0.11	60	0
IV	13.0	0.04	0.14	75	0
Va	13.9	Very low.		243.3	0
VIa	29.5	0.36	0.42	16.25	0
Vb	29.0	0.30	0.39	2.0	36.0
VIb	6.5	0.39	0.53	8.1	7.5

Concentration of Lipase by Volhard's Method.—Since much of the activity of gastric lipases is expressed in terms of Volhard's method, especially as modified by Stade, it seemed of interest to measure our juices by the same standard. The method in brief is this: I. 20 cc. of a substrate consisting of three egg yolks beaten with 100 cc. of H_2O were incubated with the enzyme mixture for 2 hours at $40^\circ C.$, at the end of which time the mixture was dried and extracted by the Soxhlet method. Alcohol was added to the ether extract and the free fatty acid titrated (phenolphthalein as indicator). II. This solution was then saponified and the total fatty acid estimated. The per cent of fatty acid split was calculated by the formula $\frac{1.100}{I + II}$. Controls were run, using boiled enzyme exactly as above. The per cent of fatty acid in the control was subtracted from that in the first case, thus giving the per cent of acid split by the enzyme. The Stade modification of this method was the shaking out of the digestion mixture with ether and titrating the fatty acid—No. 1—before saponification; and No. II—after saponification. Calculation was the same as above. In the reported work by Volhard and Stade they used fluids obtained from test meals and also extracts of the gastric mucosa. It is therefore difficult to compare their results with those we have found, since the amount of actual gastric juice present in a given volume of a test meal is unknown. Some comparison is, however, desirable and to make this, 1 cc., our routine test quantity, of clear juice was introduced into the substrate. The per cent of splitting by the Volhard method was found to be 23.2 per cent and by the Stade modification, 22.1 per cent.

Origin of the Lipase.—This much seems to be clear, that the source of the lipase is influenced by the secretory activity of the stomach. The total number of lipase units secreted rises with activity, since the concentration per unit volume falls very little over that of the fasting juice while the quantity of juice is enormously increased. Such a fact enables us to eliminate an origin analogous to mucus, but forces us to consider whether we are dealing with an excretion or secretion.

Blood Serum.—On examination, the concentration of lipases in serum by our method showed a value of 15.7 cc. of 0.05 N NaOH (average of four estimations). It will be seen that this value is about one-fifth of that found in the gastric juice which has been immediately neutralized. Carlson and Ryan (18) have shown that in the case of the cat's saliva diastases are to be found, and they regard these as filtered from the blood stream. A similar state exists in the secretion of urinary diastase from the serum (King, 19, and others), yet an abundance of evidence exists to show that

an excretory organ can separate a fluid of higher out of one of lower concentration; viz., urea in the case of the kidney, and ammonia in the case of the gastric juice (Huber, 20). One must remember, however, that these classes of substances do not include enzymes. To settle the question finally one should study the properties of the lipases of each fluid and thus their identity or difference could be conclusively established. Analogy, however, is rather against the view of the origin from the blood stream.

Origin from the Mucosa.—Greene (21) has studied fat absorption in the King salmon and finds that on feeding olive oil, fat is absorbed in appreciable quantities from the pyloric portion of the stomach. The distribution of fat in these cells is not in keeping with the view that they are storage depots. Viewing the absorption process in the stomach as similar to that in the intestinal cells, he finds here a definite argument for the existence of a gastric lipase.

In order to see what concentrations of lipases are separated by intestinal cells, which are *par excellence* cells of absorption and which therefore have an optimum concentration of intracellular enzyme, a study of succus entericus was made. Juice from a Thiry fistula, isolated from the upper jejunum, collected under widely different intestinal food states, gave a constant lipase content of 15.87 cc. of 0.05 N NaOH (average of seven estimations). This value, it will be noticed, is the same as that in the serum and only one-fifth of that of gastric juice. Values of 80 to 90 cc. of 0.05 N NaOH (gastric juice) appear rather high to be explained as by-products which the cells lose either in the performance of their own metabolism or the function of absorption.

If, then, the gastric lipase is a secretory product of physiological value we must picture to ourselves some mechanism by which it may escape destruction.

Pavlov (22) throughout his studies has contended that the cells of the stomach have but one level of HCl acid partition and that is a maximum one, in the neighborhood of 0.5 per cent, and that the lower values are due to secondary neutralization. This view has been more recently supported by Rehfuess and Hawk (23). On the contrary, Foster and Lambert (24), by studying the total chlorides, and Carlson (25), who studied the "continuous secre-

tion or hunger juice," present evidence that the gastric mechanism is capable of working at different levels. If the Pavlov view be true, then we must conceive that the time interval during which this juice of high acidity acts upon the lipase is too short for the destruction of the enzyme. We are more inclined to view the actual recovery of the enzyme from the stomach contents as shown in our experiments as another argument that the Pavlov view is incorrect.

As one looks over the possible origins of the gastric lipase, the view that we have an enzyme which is secreted as the other digestive enzymes seems to harmonize best with our experimental evidence.

Rôle of the Gastric Lipase in Digestion.—What is the importance of this gastric lipase in digestion? It has usually been assumed that, even admitting its presence in the stomach, the work accomplished cannot be relative in comparison with the ordinary pancreatic digestion. This is possibly true.

The persistence of lipase in the stomach certainly seems to be a function of the free acidity. An acidity of 0.2 per cent of HCl appears to be about the upper limit for the recognition of mucosal lipase, but in clinical observation this free acidity is not soon reached in the normal stomach under the usual diet conditions with average amounts of protein present to hold the acid. It is therefore highly probable that, before the acid can accumulate to the point where it can more than saturate the proteins, the lipase enzyme will have an opportunity of doing appreciable work, as the ptyalin of the saliva certainly does and as administered trypsin must do also, as pointed out in the investigation from this laboratory presented by Long (16). From the data at hand it is not possible to state just what the relative importance of this gastric lipolytic activity is, but it is evident that in estimating the general work of the stomach, it should not be overlooked.

The authors wish to acknowledge their obligations to Professor Long and McGuigan for assistance and suggestions in the pursuit of these studies.

SUMMARY.

1. The occurrence of gastric lipase was studied in pure juice obtained from dogs with Pavlov stomachs and those with pylorus ligated. The secretion was excited by gastrin bodies and food.

2. The fasting and acid-free juice always contains an appreciable concentration of lipase.

3. The lipase is quite sensitive to acid and alkali, being almost completely destroyed by a 15 minute exposure to 0.2 per cent HCl.

4. The enzyme may be recovered from the stomach showing low acid secretion by neutralizing the juice immediately; and from stomachs in high secretory activity, if the acid be reduced by the addition of protein, such as peptone.

5. In a series of trials 1 cc. of fasting juice gave a fat-splitting of 28.2 per cent by the Volhard method and 22.05 per cent by the Stade modification.

6. The concentration of the enzyme in the gastric juice is five or six times that in the succus entericus and the blood serum.

7. The view that the lipase is a true gastric secretory product is discussed and favored.

8. The probable practical importance of the lipase in stomach digestion is emphasized.

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AN ADAPTATION OF WINKLER'S METHOD TO BIOLOGICAL WORK.

BY W. J. V. OSTERHOUT AND A. R. C. HAAS.

(From the Laboratory of Plant Physiology of Harvard University, Cambridge.)

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Winkler's method for the determination of oxygen in solutions has been extensively employed in biology. The chief obstacles to a successful application of the method are:

1. The necessity of removing the organisms from the solution before adding the reagents. In doing this oxygen may be admitted in spite of all precautions. If the other alternative be adopted, namely, siphoning off a sample of the solution, the danger of contamination with oxygen still exists.

2. The desirability of taking samples from time to time in order to follow the course of the reaction by which oxygen is produced (as in photosynthesis) or consumed (as in respiration).

In order to obviate these difficulties the writers have employed the apparatus shown in Fig. 1. *A*. It consists of two Pyrex (or "Nonsol") glass tubes *G* and *H* (each about 30 cm. long and of about 27 mm. diameter) joined by a piece of flexible rubber tubing *F* (all the rubber tubing and rubber stoppers used in the apparatus should be repeatedly boiled and coated with paraffin) and furnished with rubber stoppers at the free ends.

One of the rubber stoppers is connected with a series of smaller glass tubes (*B*, *C*, *D*, *E*) of not less than 13 mm. inside diameter, with only enough space between them to permit the insertion of a clamp.

The procedure is as follows. The larger tubes are filled with the solution containing the organisms by pouring through *E*; or, if the organisms are too large to pass through *E*, they may be placed in the large tubes before they are joined by the flexible rubber tubing *F*. In the case of marine algæ the material may be so large that when once placed in the tube it cannot easily be

moved (by inverting or jarring the apparatus). In such case care should be taken to place the material in the large tube *G* so that when the clamp *K* is applied at *F* none of the material protrudes into *H*.

The amount of solution placed in the apparatus is carefully measured. The liquid is poured in until the level reaches the clamp between *B* and *C*. Small glass beads covered with paraffin may be placed in the solution to act as stirrers.

After closing the clamp between *B* and *C* (avoiding the inclusion of air) the apparatus is left until the determination of oxygen is made. This is done in the following manner. The apparatus is inverted several times in order to mix the contents thoroughly. This may be facilitated by attaching the entire apparatus to a piece of board. The organisms are collected in *G* (by gravity, assisted by shaking, or by means of a device to be described later); *G* and *H* are pulled slightly apart, causing the rubber tube *F* to collapse slightly. The clamp *K* is then used to close *F* completely (the jaws of the clamp are prevented from cutting *F* by strips of soft leather). The tube *G* is then removed and the volume of solution in it is measured.

The clamps above *B* are opened and alkaline potassium iodide¹ is poured into *C* until the level appears in *D*. The clamp between *C* and *D* is then closed; the excess of reagent is poured off and the tubes *D* and *E* are thoroughly rinsed. The solution of manganese chloride is poured into *D* until the level is seen in *E*. The clamp between *D* and *E* is then closed; the excess of reagent is poured off and the tube is thoroughly rinsed. (The tube *D* is about as long as *C*.) The clamp between *B* and *C* is then opened; the apparatus is inverted several times to mix the reagent with the solution in *H*.

The tube *E* (which is about three times as long as *D*) is filled with concentrated hydrochloric acid, and clamped off (so as to include no air); the excess of reagent is poured off and the tube rinsed. The clamp at the free end of *E* should be closed with special care to prevent the sucking in of air.

The clamp between *C* and *D* is now opened and the apparatus

¹ The amount will depend on the strength of the reagent and the quantity of oxygen present, and must be regulated by varying the size of *C* and the length of the rubber connection.

inverted several times to mix the reagents (the rubber connections should be squeezed, if necessary). When the mixing is **finished** the clamp between *D* and *E* is opened and the apparatus **allowed** to stand (*E* being above) without inverting (squeezing the **rubber** connections if necessary). The acid falls and mixes

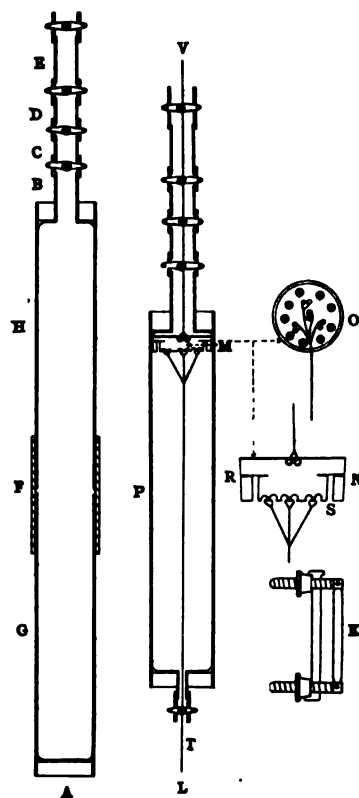


FIG. 1. Apparatus for determining the amount of oxygen in solution by Winkler's method.

by its own weight, dissolving the precipitate in the tube. When this is accomplished the clamp at the free end of *E* is opened and the liquid is poured out into a beaker. The tube *H* is rinsed with distilled water which is then poured into the beaker. Titration is then performed in the usual manner. The volume of solution

is found by subtracting the volume in G from the total amount placed in the apparatus at the start.

The writers have found it advisable in most cases to use 0.05 N sodium thiosulfate (containing 4 cc. of 1 M sodium hydroxide per liter) in place of 0.1 N as usually recommended.²

A control is made in exactly the same manner except that the organisms are absent.

The essential feature of this apparatus is the provision for collecting all the organisms into one of the large tubes while the oxygen in the other tube is determined. In some cases the organisms cannot be collected into one tube by gravity (assisted by shaking). In this case the apparatus shown in Fig. 1, L , is used. The essential feature is a piston M to which are attached strings, of small diameter but sufficiently strong, by which it can be pulled through the tube P , sweeping the organisms with it, so as to collect them in one end of the tube.

As shown in Fig. 1, N , the piston consists of a rubber stopper (snugly fitting the tube P). A circular cut R is made parallel to the plane surface of the stopper and holes S, S are bored as far as the cut. When the stopper is pulled through the tube in the direction of the letter S in the figure, the solution passes through the holes S, S , passes out through the cut (which opens under the pressure), and slips between the piston and the glass. The ease with which the liquid slips past the piston depends on the width of the flap and the taper of the stopper. If necessary, bolting cloth may be placed over the holes S, S to strain out very small organisms. The number of holes may be varied. Fig. 1, O , shows a convenient arrangement (the holes are shown in solid black).

The strings may be attached in any convenient manner. The arrangement shown in Fig. 1, O and N , is obtained by boring holes in pairs to a moderate depth and puncturing the connection between each pair so as to admit the string.

When the apparatus is set up the plunger M is placed as shown in the figure. Solution and glass beads are placed on both sides

² Treadwell, F. P., Analytical Chemistry, translated by Hall, W. T., New York, 4th edition, 1915, ii, 760. For directions for making the dilute solutions see Birge, E. A., and Juday, C., *Wisconsin Geol. and Nat. Hist. Survey, Series 7, Bull. 22*, 1911, 13-21.

of *M* and the clamps closed. When the oxygen is to be determined the organisms are swept out of the tube by pulling the plunger as far as possible toward *T* (without opening the clamp). The plunger is then pulled back (toward *V*) just enough to straighten the flap of the stopper so that it fits the tube snugly and prevents the passage of liquid.

In a special case it might be possible to place the tube on a centrifuge (the end at *T* being fused instead of closed by a stopper). The organisms would then be collected by centrifugal force at the end *T* and the piston would automatically follow them.

The solution, freed of organisms, is treated as previously described. The volume is easily ascertained by pouring in liquid through the end (at *V*) until it reaches from the piston (in its final position) to the first clamp.

It is of course desirable to invert the apparatus frequently during the experiment so as to mix the contents of the tube. (The first small tube above *M* need not be large enough to introduce an appreciable error due to the fact that the liquid in it does not come into contact with the organism. This error is, in any case, a constant one.)

In some cases it is desirable to follow changes in the reaction of the solution. For this purpose an indicator may be added to the solution at the start. This must be such as not to injure the organisms or interfere with the determination of oxygen (phenolphthalein may be recommended for this purpose). The color of the solution may then be observed at any time (preferably through the small glass tube *B*) and compared with buffer solutions (of known hydrogen ion concentration) contained in tubes of the same diameter and containing indicator of the same concentration.³

In order to take samples during the progress of an experiment the tubes *G* and *H* are replaced by large T-tubes which are connected end to end (several may be placed in series, attached to a board, so as to be easily handled). A set of small tubes (*B*, *C*, *D*, *E*) is attached to each T-tube by its side arm. Whenever it is desired to make a determination the T-tube at the end is

³ Haas, A. R., *Science*, 1916, xliv, 105.

clamped off (after clearing it of organisms) and the usual procedure is followed.

Allowance must be made for the fact that after any T-tube is clamped off the organisms are in a smaller volume of liquid. As the oxygen content is known this presents no difficulty.

The simplest method of following a reaction is to renew the solution completely after each determination is made. If for example the effect of an anesthetic on oxygen consumption is to be determined a considerable quantity of the solution containing the anesthetic is made up at the start. Some of this is placed in the apparatus shown in Fig. 1, *A*. When the tube *H* is clamped off at *F* the organisms in *G* are rinsed in a fresh lot of the solution either by allowing them to settle and then decanting or by pushing down the piston *M* into the tube, pouring out the liquid, adding fresh solution, and pulling out the piston. The tube *H* is then replaced by a similar tube, which is joined to *F*. The whole apparatus is filled with fresh solution and the usual procedure followed.

In this way the supply of oxygen is kept up by frequent renewal and determinations may be made as frequently as desired.

SUMMARY.

The adaptation of Winkler's method here described permits:

1. Removal of the organisms before adding the reagents.
2. Adding the necessary reagents without danger of contamination by oxygen.
3. Taking samples at intervals during the experiment.
4. Continuous observation of changes produced in the reaction of the solution by such processes as respiration and photosynthesis.

THE DIFFUSION OF ELECTROLYTES THROUGH THE MEMBRANES OF LIVING CELLS.

V. THE ADDITIVE EFFECT OF SALT AND BASE AND THE ANTAGONISTIC EFFECT OF SALT AND ACID.

By JACQUES LOEB.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

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I. Introductory Remarks.

In former papers¹ it has been shown that the washed membranes of the egg of *Fundulus* are impermeable for potassium salts, that the addition of a moderate amount of a second salt accelerates the diffusion, and that an addition of a greater quantity of a second salt inhibits the diffusion of potassium salts into the egg. When alone in solution potassium salt cannot diffuse through the membrane of washed eggs until the potassium salt itself has supplied the "salt effect." It was pointed out that these effects of salt on diffusion were analogous to the effects of salts on globulins, which are insoluble in pure water, soluble in a moderate concentration, and insoluble again in a very high concentration of salt.² This would make it appear as if the diffusion of potassium salts depended on the solution of a certain membrane constituent, with qualities resembling those of a globulin. Potassium was used in these experiments since it causes cessation of the heart beat of the embryo as soon as a certain quantity has diffused into the egg. The cessation of the heart beat was, therefore, a convenient indicator for the diffusion of a certain quantity of potassium into the egg.

It seemed desirable to test whether the analogy between the behavior of globulins and the diffusion of electrolytes through

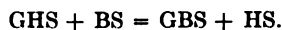
¹ Loeb, J., *J. Biol. Chem.*, 1916, xxvii, 339, 353, 363; 1916-17, xxviii, 175.

² Loeb, J., *J. Biol. Chem.*, 1917, xxxi, 343.

the membrane could be carried further, and for this purpose the effect of neutral salts on the diffusion of acids and alkali was examined. Hardy states that there exists an antagonism between the solvent actions of salts and acids while the solvent actions of salt and base are additive. From this difference Hardy has drawn interesting inferences on the manner of combination between globulins, salts, acids, and alkali.

"There is however one feature of fundamental importance which is never obscured, and that is the antagonism between the solvent actions of salts and acids, and the additive nature of the combined solvent action of salts and alkali. This feature arises I believe from the fact that acid globulin is insoluble by salt. Salts will combine with globulin or with alkali globulin to form soluble compounds; they will not so combine with acid globulin.

If this be true, then, when acid globulin is precipitated and redissolved by salt, the acid must be displaced and the globulin redissolved, not as salt-acid globulin but as salt globulin. The displacement of the acid can readily be followed by methyl orange. When HCl globulin is precipitated by a salt this indicator shows that free acid is liberated. But I failed completely to detect the liberation of free alkali when salt is added to a solution of alkali globulin. This suggests that precipitation of acid globulin is partly a definite chemical replacement of acid by salt,



The fact that alkalies slightly assist solution in salts while acids very generally depress it suggests an interesting possibility, namely, that in the compounds GHS and GBS the acid HS and the salt BS are united to the molecule of G in the same way, so that they compete with one another, while in GB and GHS the base and salt are united to different parts of the molecule."

It was, therefore, expected that if the analogy between the action of electrolytes upon the solution of globulin on the one hand, and the diffusion through the membrane on the other, would hold, that the addition of a salt should increase the rate of diffusion of alkali into the egg and diminish the rate of the diffusion of acid into the egg. This should show itself in this way, that a neutral salt would increase the toxicity of a base and diminish the toxicity of an acid. This is actually the case, as will be shown in this paper. It was necessary to make sure that the salts used were neutral or at least not alkaline, since

² Hardy, W. B., *J. Physiol.*, 1905-06, xxxiii, 323-24.

if they were alkaline the additions of salt to acid would make the acid weaker and when added to alkali increase the alkalinity. This would indeed result in an apparent antagonism of salt to acid and an apparent additive effect of salt to base. Hence to prove a real antagonism between acid and salt it was necessary to make sure that the salt solutions were not alkaline. Tests were made with phenolphthalein, neutral red, and congo red. The neutral red tests showed that the salt solutions used (with the exception, of course, of sodium citrate) were all slightly on the acid side of neutrality (all being as red as or more than distilled water). A trace of alkali sufficed to bring them to neutrality. Thus, one drop (0.05 cc.) of $N/100$ NaOH sufficed to turn 50 cc. of $M/4$ NaCl or $M/32$ $CaCl_2$ or $M/32$ $SrCl_2$ from red to yellow (with neutral red as an indicator). Na_2SO_4 was more decidedly acid, 0.5 cc. $N/100$ NaOH being required to turn 50 cc. $M/32$ Na_2SO_4 yellow. We are, therefore, dealing in the following experiments with an effect of the neutral salt.

The addition of a neutral salt to an acid with different anion has no effect on the hydrogen ion concentration. Dr. Haas was kind enough to compare with the aid of his indicators the H ion concentration of $M/500$ acetic acid made up in H_2O and $M/32$ Na_2SO_4 , and found it identical, namely 3.6×10^{-5} .

II. *The Antagonistic Character of the Combined Action of Salts and Acid.*

Loeb and Wasteneys⁴ have published two papers showing that the adult fish of *Fundulus* can resist acid much better if some neutral salt (NaCl or $CaCl_2$) is added to the acid. Acid killed these fish by altering the surface of their gills whereby respiration became impossible. The effect of the acid on the external surface of the fish was directly visible inasmuch as the outer lining of the body became white and peeled off. The presence of an adequate amount of salt prevented this effect of the acid when the latter was not too concentrated.

2 years ago the writer published similar experiments on the effect of neutral salt on the rate of diffusion of acid into the egg

⁴ Loeb, J., and Wasteneys, H., *Biochem. Z.*, 1911, xxxiii, 489; 1912, xxxix, 167.

of *Fundulus*.⁵ The acid had to diffuse through the membrane in order to reach the embryo. As soon as the acid penetrates in sufficient quantity into the egg the embryo begins to coagulate and become white, the thinnest part, the tail, showing this effect earlier than the rest. The heart is protected by the pericardium and therefore the heart beats may still be going on at a time when the tail is already coagulated by the acid. As soon as the heart stops beating recovery is no longer possible, since by this time the whole body is coagulated and for this reason the cessation of the heart beat could in this case be used as the criterion for death.

It was found that neutral salts inhibit the rate of diffusion of acid through the membrane. This could be proved by the fact that rhodanates and tartrates prevented the toxic action of acid upon the embryo *inside* the egg, while these salts had no such action upon the fish when outside the egg, the salts themselves being too toxic to be of any use for the fish itself. They were, however, of use in acting upon the external surface of the membrane of the egg, thereby retarding the diffusion of acid through the membrane.

The inhibiting action of salts on the rate of diffusion of acid through the membrane is a function of the anions as well as of the cations. Organic sodium salts antagonize acid better than the inorganic ones and the bivalent anions better than the monovalent ones. Likewise Ca and Sr antagonize better than the univalent cations and also better than Mg and Ba.

The relative efficiency of cations in antagonizing acid is shown in the following table, giving the *minimal* concentration of different neutral salts which permits 50 per cent of the embryos to survive in M/500 acetic acid after 18 hours.

LiCl.....	Die in all concentrations.
NaCl.....	" " " "
RbCl.....	" " " "
CsCl.....	" " " "
MgCl ₂	" " " "
CaCl ₂	M/2048
SrCl ₂	M/512
BaCl ₂	M/32

⁵ Loeb, *J. Biol. Chem.*, 1915, xxiii, 139.

The superior antagonistic effect of the bivalent over the univalent cations is beyond doubt, as is also the difference between Ca and Sr on the one hand and Mg and Ba on the other.

As far as the anions are concerned, the difference in the antagonistic action between Cl and SO₄ is marked and independent of the nature of the cation. Table I gives the percentage of surviving embryos in M/500 acetic acid in M/8 chlorides and sulfates of different cations.

TABLE I.

After.	Percentage of surviving embryos in M/500 acetic acid made up in chlorides and sulfates of different cations.		
	Cation.	Cl	SO ₄
hrs.			
24	Li	0	80
24	Na	0	100
8	NH ₄	0	80
24	Mg	0	100

The sulfates are so much more powerful in their antagonism to acetic acid than the chlorides that at the time when the embryos had all been killed in the chloride solution they were still all active and alive in the sulfate solution.

In order to get a quantitative expression of the relative efficiency of univalent and bivalent anions the embryos were put into M/500 acetic acid solution to which various concentrations of different sodium salts were added. That concentration of these salts was ascertained which allowed 50 per cent of the eggs to be alive after 10 hours. This concentration was found to be for

NaCl.....M/4
 NaBr.....M/4
 NaNO₃.....Slightly > M/4
 Na₂SO₄.....M/32

When we add increasing quantities of salt to an acid very soon a concentration is reached where the further addition of salt accelerates the action of the acid; this action takes place at a concentration of the salt where the salt alone is not yet very harmful.

TABLE II.

	Percentage of surviving embryos after 6 hrs. in m/500 acetic acid made up in										
	m/1	m/2	m/4	m/8	m/16	m/32	m/64	m/128	m/256	m/512	m/1024
NaCl.....	0	40	100	75	35	5	5	0	5	0	0
NaBr.....	50	90	95	50	10	0	0	15	5	0	10
NaNO ₃	15	95	95	40	20	15	5	0	0	10	0
Na ₂ SO ₄		90	100	100	100	100	80	80	10	5	10

TABLE III.

	Percentage of surviving embryos after 10 hrs. in m/500 acetic acid made up in										
	m/1	m/2	m/4	m/8	m/16	m/32	m/64	m/128	m/256	m/512	m/1024
NaCl.....	0	10	60	30	0	0	0	0	0	0	0
NaBr.....	15	85	70	0	0	0	0	0	0	0	0
NaNO ₃	0	80	40	5	0	0	0	0	0	0	0
Na ₂ SO ₄		50	100	100	100	90	10	0	0	0	0

Especially after 10 hours it is clear that the antagonistic effect of m/1 solutions of NaCl, NaBr, and NaNO₃ is less than that of m/2 or m/4 solutions. When the salt concentration exceeds a certain limit its antagonistic action to acid diminishes again.

In a previous paper it was pointed out that when eggs are treated with a low concentration of salt and are afterwards exposed to acid the diffusion of acid may be accelerated. Such an effect was never observed when acid and salt were simultaneously in solution no matter how low the concentration of the salt.

III. The Additive Character of the Combined Action of Alkali and Salt.

In working with alkali we have to remember that the CO₂ of the air as well as that produced by the living organism steadily diminishes the concentration of the alkali. This makes it necessary to carry on these experiments in small closed Erlenmeyer flasks in which the volume of solution is large compared

with the air space left and the volume of the eggs (20 in each flask). In addition it is necessary to restrict the experiment to short periods. At given intervals the eggs were taken out and examined and the percentage of embryos whose heart was still beating was determined. It was found that the salt always increased the efficiency of the alkali and in no case antagonized it. The following experiments with $m/200$ NaOH and $m/200$ tetraethylammoniumhydroxide may serve as an example. The solutions were made up in mixtures of NaCl, KCl, and $CaCl_2$ in the proportion in which these three salts exist in the sea water; namely, 100 molecules of NaCl to 2.2 molecules of KCl to 1.75 molecules of $CaCl_2$ (Table IV).

TABLE IV.

Solution.	Percentage of eggs surviving after 5 hrs. in various alkali solutions made up in NaCl + KCl + $CaCl_2$.									
	m/1	m/2	m/4	m/8	m/16	m/32	m/64	m/128	m/256	m/512
$m/200$ NaOH.....	0	0	0	10	30	45	80	100	100	100
$m/200$ tetraethylammoniumhydroxide.....	0	0	0	0	0	10	10	45	90	100

Even as low a salt concentration as $m/32$ has an accelerating effect upon the toxic action of alkali. It may be well to point out that these eggs can live for days in neutral solutions of NaCl + KCl + $CaCl_2$ as concentrated as 2.5 m .

In order to ascertain whether at least a trace of an antagonistic effect of salts upon alkali can be discovered, higher concentrations of the latter were used. The writer did not observe any indication of an antagonistic effect between a strong base and salt while the additive effect was always marked.

The next task consisted in ascertaining the influence of valency and nature of anions and cations of the salt on the additive effect. It was found that the same ions which had the greater antagonistic effects upon acid had the greater additive effect upon bases; i.e., it required less of sulfate than of a chloride to accelerate the toxic effect of a base and less Ca than Na. In order to test this $m/400$ solutions of ethylamine were used. In a pure $m/400$

ethylamine solution the embryos live at least for a number of days. If we add, however, a neutral salt the same concentration becomes extremely toxic. If different sodium salts are added the toxicity of the solution increases with the valency of the anion, approximately in harmony with Hardy's exponential law. Table V gives the percentage of surviving embryos in $M/400$ ethylamine when made up in solutions of $NaCl$, Na_2SO_4 , Na_2 tartrate, Na_2 citrate, $CaCl_2$, and $SrCl_2$ after about 22 hours.

TABLE V.

Solution.	Percentage of surviving embryos after 22 hrs. in $M/400$ ethylamine made up in various concentrations of salt solutions.									
	$M/16$	$M/32$	$M/64$	$M/128$	$M/256$	$M/512$	$M/1024$	$M/2048$	$M/4096$	$M/8192$
$NaCl$	30	50	75	70	75	70	95	90		95
Na_2SO_4	25	30	20	65	80	80	95	95		95
Na_2 tartrate.....	40	20	25	50	65	80	75			95
Na_2 citrate.....	0	0	0	10	5	30	70	80		90
$CaCl_2$	5	5	0	5	20	30	50	60	85	100
$SrCl_2$	25	30	30	35	55	100	85	95	85	90

Comparing the concentration of these salts which permits 50 per cent of the embryos to remain alive we find this to be for

$NaCl$	$M/32$
Na_2SO_4	$M/128$
Na_2 tartrate.....	$M/128$
Na_2 citrate.....	$M/1024$

The ratio of efficiency of monovalent to bivalent to trivalent anion is, therefore, as 1:4:32, while Hardy's rule demands in this case 1:4:16. The exception in citrate is probably due to the fact that citrate is in itself alkaline. In experiments of shorter duration the ratio between tartrate, sulfate, and citrate was $M/128$: $M/128$: $M/512$, which shows citrate to be four times as efficient as sulfate. In the writer's previous papers it was pointed out that Hardy's valency rule holds also for the influence of salts on the rate of diffusion of KCl into the egg.

As far as the cations are concerned, it is obvious that the addition of a slight amount of $CaCl_2$ accelerates the action of the

base considerably more than NaCl, $m/1024$ CaCl_2 being as efficient as $m/32$ NaCl. SrCl_2 is slightly less efficient than CaCl_2 .

It is of interest that the addition of salts to NH_4OH gave no clear results; there was neither a clear additive effect nor an antagonistic effect between salt and NH_4OH .

IV. Proof That the Salt Action Described in This Paper Is Due to an Influence upon the Rate of Diffusion by Alkali and Acid Through the Membrane.

As stated in previous papers the experiments on the *Fundulus* egg have the striking advantage of demonstrating directly whether a salt action is due to an influence upon the rate of diffusion through a membrane or whether it is due to an action on the living protoplasm. The embryo lives inside the membrane of the egg from which it is separated by a watery liquid. By comparing the effect of a solute on the embryo while in the egg, and on the embryo immediately after hatching, we can make sure whether or not the effect observed on the egg is merely due to an influence upon the rate of diffusion through the membrane or to an effect upon the embryo inside the egg. When the difference in the two cases is merely one of degree a doubt might still be entertained; but when it becomes one of kind, as is the case in these experiments, all doubt vanishes.

The *Fundulus* embryo inside the egg remains alive indefinitely when the eggs are put into $m/400$ ethylamine but they all die in less than 24 hours when the $m/400$ ethylamine is made up in $m/64$ CaCl_2 or in some other salt. The salt accelerates death. When we make the same experiment on the newly hatched embryo we find that the latter dies in about 20 minutes in $m/400$ ethylamine and the addition of CaCl_2 does not accelerate the action but retards it markedly, the embryo living from 40 to 60 minutes in $m/400$ ethylamine made up in $m/32$ CaCl_2 . The same is true for other salts, e.g., Na_2SO_4 or SrCl_2 ; instead of accelerating the action of alkali on the hatched embryo they retard it. This effect of the salt then is exactly the reverse from the effect of the salt upon the embryo while inside the egg. This does not permit of any other interpretation than that the combined effect of salt and alkali upon the egg is due to an action

on the membrane surrounding the egg and not upon the embryo inside the egg. We must therefore conclude that the addition of a neutral salt to the alkali allows the latter to diffuse more rapidly into the egg. The embryo which is killed in 20 minutes in $m/400$ ethylamine, when brought directly into this solution, remains alive in this solution, as long as it is surrounded by the egg membrane, and the reason can only be that this solution never reaches the embryo as long as it is surrounded by the membrane. If, however, a salt of the proper concentration is added to the $m/400$ ethylamine solution the latter can diffuse into the egg. In a stronger ethylamine solution, *e.g.*, $m/200$, the embryos are all killed inside of 24 hours when inside the egg. In this case the higher concentration of the alkali itself supplies the "salt effect" upon the diffusion of base into the egg.

As far as the direct action of alkali on the embryo is concerned it is visibly due to the solution of the surface elements of the embryo. In a $m/400$ ethylamine solution the surface of the head is dissolved very quickly, leaving the eyes protruding.

We have already mentioned the fact that tartrates and rhodanates can be used to antagonize the action of acid on the egg, while they cannot be used to counteract the injurious effect of acids on the fish when outside the egg.⁵ This shows that the antagonistic action of salt to acid, as long as the embryo is inside the egg, is due to an effect on the membrane of the egg and not on the embryo itself.

V. Theoretical Remarks.

We have pointed out in this and in a previous paper the analogy which exists between the conditions for the diffusion of electrolytes through the membrane of the *Fundulus* egg and the condition for the solution of globulins. It seems on the whole that those conditions which tend to make globulins soluble also permit or accelerate the diffusion of certain electrolytes through the membrane of the egg of *Fundulus*; while those which render globulins less soluble also inhibit or retard the diffusion of certain electrolytes. Thus it was shown in a former paper that potassium salts diffuse through the membrane of *Fundulus* when the membrane has had a chance to combine with

a certain moderate quantity of salt; while either a membrane free from salt or one having an excess of salt is more or less impermeable for the same potassium salt. This corresponds to the fact that globulins are soluble in a moderate amount of salt but insoluble in distilled water or in an excess of salt. It was also pointed out that the valency rule found by Hardy for the precipitation of globulins holds also for the action of salt on the diffusion of potassium through the membrane of the *Fundulus* egg. It was pointed out, however, that there exists merely an analogy and not an identity in the diffusion of salts through the membrane and the solubility of globulins; since the ratio for the solvent and precipitating effect of a neutral salt on globulins differs widely from the ratio for the accelerating and inhibiting effect of the same salt for the diffusion through the membrane.

In this paper the analogy is carried further. Salts and acids have an antagonistic and salt and bases an additive effect upon the solution of globulins. It could be shown that the relation holds for the diffusion of acid and bases through the membrane. Salts accelerate the diffusion of bases and retard the diffusion of acid through the membrane. The analogy apparently breaks down again through the fact that the increasing valency of both cations and anions of salts increases the antagonistic action to the diffusion of acid and increases the accelerating action on the diffusion of alkali.

Salts, therefore, increase the rate of diffusion of certain electrolytes through the membrane of the egg of *Fundulus* when they are also able to dissolve globulins; and they retard or prevent the diffusion when they are likely to prevent the solution of globulins. The facts, however, do not permit us to say that the substance in the membrane upon which the diffusion of electrolytes depends is a globulin. A difference in the action of salts on acids and bases was also observed by the writer in his old experiments on the swelling of muscle, where he found an antagonistic action of neutral salts to acid but not to base.⁶

Some authors assume that the proof of an increase in permeability of a membrane to one substance indicates an increase of permeability to all substances. The fact that salts increase

⁶ Loeb, *Arch. ges. Physiol.*, 1899, lxxv, 303.

the permeability of the *Fundulus* membrane to alkali while they have the opposite effect on the permeability to acids should warn us against such generalizations.

SUMMARY OF RESULTS.

1. When *Fundulus* eggs are put into solutions of bases not sufficiently concentrated to injure the embryo the bases become injurious when neutral salts in low concentration are added. This additive effect between salts and bases was observed also at higher concentrations of the base.

2. When *Fundulus* eggs are put into an acid which kills the embryo rapidly the addition of a neutral salt has an antagonistic effect. The same antagonism between acid and salt was observed also for lower concentrations of acids.

3. The minimal quantity of a salt required for these effects diminishes with an increase in the valency of both anion and cation of the salt. In the case of the anion the quantity diminishes according to Hardy's valency rule. In addition the chemical nature of the salt is of importance.

4. It can be shown that the additive effects of salt to base and the antagonistic effect of salt to acid are due in the case of the egg of *Fundulus* to an influence of the salt upon the rate of diffusion of alkali and acid through the membrane of the egg; this influence being accelerating for the diffusion of alkali and retarding for the diffusion of acid.

5. The partial similarity of the action of neutral salts in these cases with the action of salt on the solution of globulins in the presence of acid and alkali is pointed out.

THE DETERMINATION OF SMALL AMOUNTS OF CALCIUM, PARTICULARLY IN BLOOD.

By JOHN O. HALVERSON AND OLAF BERGEIM.

(From the Laboratory of Physiological Chemistry of Jefferson Medical College, Philadelphia.)

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The determination of calcium in small amounts of blood and other biological materials low in this element involves the solution of three main problems. The first is the preparation of a solution containing all of the calcium but free from interfering organic matter. The second is the precipitation of the calcium in the presence of such other elements as commonly occur, while the third consists in the accurate estimation of this precipitated calcium.

Toward a solution of these problems the present authors have suggested the precipitation of calcium as oxalate directly in the filtrate from biological fluids after removal of protein, the final estimation being carried out by a refinement of the permanganate titration method (1). In this paper is presented in greater detail the procedure which our experience with this method has suggested as well as data with regard to the accuracy of estimation. It is our belief that in many cases the substitution of deproteinization for ashing marks a distinct advance, and that evidence as to the accuracy with which minute amounts of calcium may be estimated by permanganate titration will show this titration to have a wider applicability than that with which it has generally been credited.

Methods.

I. Procedure for Serum, Plasma, and Whole Blood.

A. Removal of Protein.—Whole blood is preserved with powdered sodium citrate to make approximately 1.5 per cent. An additional 1 per cent of citrate should be added to plasma if this

is not to be analyzed at once. Directions given below are for serum or plasma. Twice the quantity of whole blood should be employed and reagents increased proportionately.

Pipette 5 cc. of serum¹ or plasma into a 50 cc. volumetric flask containing exactly 20 cc. of distilled water. Rinse by once drawing the solution up into the pipette. While rotating the flask add from a pipette 5 cc. (1 cc. per cc. of plasma or serum) of a 4 per cent solution of sodium picrate. In the same manner add slowly 5 cc. of hydrochloric acid (1:2). Heat in a boiling water bath with occasional rotation for 15 minutes. Cool to a little below room temperature in cold water. Pour onto a folded calcium-free filter paper and allow to drain well.

B. Precipitation of Calcium.—Measure an aliquot (usually 25 cc.) of the filtrate into a 50 cc. Erlenmeyer flask (Pyrex). Neutralize cautiously with concentrated ammonium hydrate added drop by drop from a burette, using one or two drops of alizarin indicator solution (0.2 per cent). Titrate back until faintly acid with approximately 0.5 N hydrochloric acid. McCrudden's method is then followed. Add from a burette 2.5 cc. of the hydrochloric acid 0.5 N mentioned above and then the same amount of 2.5 per cent oxalic acid. To the boiling solution add dropwise in two portions 2.5 cc. of 3 per cent ammonium oxalate. Digest at near boiling for 15 minutes.

Cool in ice water to room temperature or lower. Add another drop of alizarin and also dropwise from a burette 2.5 cc. of 20 per cent sodium acetate solution while rotating the flask, or if found necessary add until the alizarin just *begins* to change color. Allow to stand over night (or at least 4 hours after 10 minutes' shaking).

Transfer completely to a 50 cc. round bottom centrifuge tube with the aid of a little water and whirl for about 3 minutes at 1,500 revolutions per minute. With an automatic siphon (Fig. 1) draw off the supernatant liquid at first rapidly and then gently to within a drop or two. Wash with cold distilled water

¹ If more than 5 cc. of serum is available the amount may be increased as the accuracy of the method increases somewhat with the amount of serum used. For instance 7 cc. of serum may be diluted to 50 cc. and 40 cc. of filtrate employed. For amounts smaller than 5 cc. the ashing procedure may be used.

(15–20°C.) first adding about 20 cc., washing down the sides and rotating the tube. Then add more water from the wash bottle to within about 1.75 cm. of the top of the tube (approximately 50 cc.). Placing the metacarpal portion of the palm of the hand at the thumb over the mouth of the tube shake vigorously for 5 to 10 seconds. Centrifuge again. Siphon off to within one or two drops and titrate.

C. Titration.—To the precipitates in 50 cc. centrifuge tubes add, with shaking, 4 cc. of 5 per cent sulfuric acid (very faintly



FIG. 1.

tinged with potassium permanganate). Place in a water bath at 65°C. until the tubes approach the temperature of the bath. Remove and titrate rapidly with an approximately 0.0133 N potassium permanganate solution, with moderate shaking using a white background. A burette of 10 cc. capacity with which readings can be readily estimated to 0.01 cc. is desirable.

The end-point is attained when a faint but definite pink color persists for a minute or longer on gentle shaking and standing. If the precipitate has not been contaminated the end-point will

be sharp to 0.01 cc. The sulfuric acid must be brought in contact with all parts of the tube as far up as the original solution extended. The burette reading should be corrected for the small amount of permanganate required to titrate 4 cc. of the sulfuric acid to the same end-point.

D. Calculation.—1 cc. of 0.0133 N potassium permanganate is equivalent to 0.267 mg. of calcium. The exact factor for a given solution must be determined by standardization. Multiply the number of cc. used by this factor to obtain the amount of calcium in the 25 cc. of filtrate. If blank on reagents is not negligible deduct. Multiply by 28 to get mg. of Ca per 100 cc. of serum or plasma.

II. Procedure for Human and Cow's Milk.

For human milk dilute 5 cc. with an equal volume of water and treat 5 cc. of the mixture in exactly the same way as serum (Procedure I, above). The deproteinization procedure is here advantageous because of the relatively low calcium content of this fluid (about 30 mg. per 100 cc.).

For cow's milk dilute 5 to 50 cc. and treat 5 cc. of the mixture in the usual manner (Procedure I). Here deproteinization has less advantage over ashing.

III. Procedure for Cerebrospinal Fluid.

10 cc. of spinal fluid in a 25 cc. volumetric flask are treated with 10 cc. of water and 1 cc. of sodium picrate solution and then 1 cc. of concentrated hydrochloric acid is added slowly with rotation. The flask is then made up to mark, heated in a boiling water bath for 15 minutes, cooled, filtered, and an aliquot, usually 20 cc., used.

IV. Procedure for Urine.

Where small amounts of urine only are available or where the highest percentage accuracy is not required the following procedure has been found advantageous.

10 cc. of filtered urine (in exceptional cases 20 cc.) are introduced into a 50 cc. Erlenmeyer flask. Concentrated urines should be diluted with an equal volume of water. The calcium

is then precipitated following exactly McCrudden's procedure (2) merely using correspondingly smaller amounts of reagents. The sodium acetate solution should be added with great care. Centrifuge and wash in the manner indicated for blood serum.

In the precipitation, by shaking 10 minutes and allowing to stand for 4 hours, there is little danger of precipitating uric acid which must be absent in the titration. If more convenient however, allow to stand over night. If uric acid separates the oxalate may usually be washed out from the flask with the aid of small portions of water, leaving the much larger and heavier uric acid crystals behind.

V. Determination in the Ash from Biological Materials.

The ash is dissolved by digesting with dilute hydrochloric acid for about 30 minutes. Neutralize with ammonia and precipitate the calcium in the usual way using amounts of reagents proportional to the volumes of the solutions.

Reagents.

Potassium Permanganate 0.0133 N.—Dissolve 0.50 gm. of pure potassium permanganate crystals in 1 liter of redistilled water in a thoroughly clean Florence flask which has been rinsed with the same water. Digest at near the boiling point for 36 hours. A funnel covered with a watch-glass may be used as a reflux condenser. Cool and allow to stand over night. Without disturbing the sediment of manganese oxides filter with gentle suction through a 3 inch Buchner funnel lined with ignited asbestos. Both funnel and flask should be rinsed with redistilled water. Transfer the permanganate solution to a glass-stoppered bottle free from traces of organic matter and keep in the dark when not in use.

This permanganate solution may be conveniently standardized against oxalic acid (0.1101 gm. pure crystals to 100 cc.) or sodium oxalate of similar strength. To 10 cc. of the oxalic acid solution (equivalent to 3.5 mg. of Ca) in a 50 cc. Erlenmeyer flask add 10 cc. of 10 per cent sulfuric acid which has been treated with just sufficient permanganate solution to give it a faint pink color. Place in a water bath at 65°C. for a few minutes. Remove and titrate at once to a definite pink color which persists for at least a minute. If kept in a dark place the oxalic acid solution does not lose appreciably in strength in from 10 days to 2 weeks. Ordinarily the permanganate solutions after they have stood for several days will not vary over 0.1 per cent a week. On account of the sensitivity of the reagent and as a control on technique it is, however, desirable to run a check with each series of determinations.

Sodium Picrate Solution, 4 per Cent.—To 40 gm. of dry purified picric acid¹ add a little calcium-free water and 10 gm. of highest purity anhydrous sodium carbonate (calcium-free) dissolved in 50 cc. of water. Dilute to 1 liter. Shake until the picric acid is completely dissolved. Add concentrated hydrochloric acid until a slight permanent precipitate of picric acid forms. Filter through highest grade filter paper.

Sodium Alizarin Sulfonate Solution, 0.2 per Cent.—An aqueous solution. There is no especial difficulty in using this indicator in the presence of picric acid.

Hydrochloric Acid, Approximately Half Normal.—Dilute 40.5 cc. of 36 per cent hydrochloric acid to a liter.

Oxalic Acid Solution, 2.5 per Cent.—Let stand over night and filter if necessary.

Ammonium Oxalate Solution, 3 per Cent.—Let stand over night. Filter off the precipitate.

Sodium Acetate Solution, 20 per Cent.—The crystallized salt is used. Filter and add a few cc. of chloroform as a preservative.

• *Sodium or Potassium Citrate, Crystallized.*—This as well as the other chemicals used must be free from all but the most minute traces of calcium. Ordinary c.p. grades must be recrystallized.

Apparatus.

In addition to the centrifuge and tubes the only special apparatus required is a simple automatic siphon of the type illustrated in Fig. 1. We have found an apparatus of this type necessary to facilitate the rapid and complete removal of supernatant liquid and wash water after centrifugation. Unless very gentle suction is employed there is great danger of loss due to disturbance of the finely crystalline precipitate. It is also necessary to draw off all but one or two drops of fluid in order to avoid an additional washing to remove the last traces of soluble oxalate.

An electric bulb is placed just above the arm A (Fig. 1) and a black cardboard extending from slightly above A to below D is attached to the right arm of A so as to leave the left arm unhampered. 4 mm. glass tubing is used. The tip of D is slightly curved as shown in the cut, aiding greatly in removing the last few drops of liquid without disturbing the precipitate. A

¹ Picric acid may be purified as follows. To 50 gm. of picric acid add 700 cc. of distilled water. Boil until clear, and while boiling add 10 cc. of concentrated hydrochloric acid. Cool. Wash by decantation with 100 cc. of distilled water. Repeat the recrystallization. Transfer to a Buchner funnel and wash with about 150 cc. of water. Dry in a desiccator.

rubber connection is used for D in order to give slight flexibility and facilitate exchange of tips. The siphon is filled by opening the upper stop-cock, and in siphoning the lower stop-cock is used to regulate the flow.

If any trace of precipitate is accidentally drawn into D or A it at once becomes visible and by closing the lower and opening the upper pinch-cock can be washed back into the tube. In a similar way the siphon may be rinsed and centrifugates separated quantitatively for further examination. The reservoir is kept filled with redistilled water preserved with chloroform. A glass stop-cock may be conveniently substituted for the lower pinch-cock.

DISCUSSION.

Citrate is used to prevent clotting because it is satisfactory and more available than hirudin. The presence of the amounts used we have found by many determinations not to interfere in any way with the precipitation of calcium. Neither is any calcium precipitated out from plasma by citrate as it is with oxalate. Diluting citrated whole blood with water to three volumes and adding a few cc. of chloroform aids in its preservation for longer periods of time. Dilution of plasma with several volumes of water after the further addition of citrate was also found useful but it did not keep as long as whole blood.

The usual gravimetric determination of calcium when applied to the ash of small amounts (25 cc. or less) of blood is inaccurate for several reasons. Aside from the limitations of weighing which under these conditions will ordinarily involve a high percentage error, it is also necessary to carefully reprecipitate the oxalate or very high results may be obtained due to occlusion, silica here causing most of the difficulty.

Another source of error lies in ashing the blood or serum, when this is carried out in the usual way. The greater the amount of blood or serum taken for ashing the more serious does this error become. The ashing of 25 cc. of blood is not only tedious but the losses of calcium are usually quite marked. By decreasing the amount of serum to 10 cc. or less, ashing is facilitated and losses are reduced. Ignition is also facilitated by evaporation in a Freas oven over night at 90-95°C. which gives a porous

protein mass readily and completely burned off by the aid of a 3 inch microburner applied carefully at one side. The ignition may also be completed in an electric oven. The hot plate should not be used as it bakes the protein into a hard mass and causes an incrustment of the salts which makes ignition difficult. Good results may be obtained by ashing small amounts of serum as indicated, but care is required and the tendency to run low must be borne in mind.

In our earlier work a preliminary washing with 0.12 per cent ammonium oxalate solution was used but this proved unnecessary as a single washing with water is sufficient to remove soluble oxalate if the centrifugates are drawn off completely as directed. This latter is, however, quite necessary as otherwise an additional washing would be required. The solubility of calcium oxalate in the wash water is negligible if centrifugation is carried out promptly. The flasks in which the precipitation is carried out and the centrifuge tubes should be scrupulously clean and bright and no dust or organic matter must be permitted to enter them.

The preparation of weak standard permanganate by dilution of stronger solutions even if the reagent is used at once leads to inaccurate results as we have pointed out elsewhere (3). A more concentrated sulfuric acid or a much higher temperature in the titration must also be avoided as these lead to high results and an indefinite end-point. Under the conditions of the method the end-point offers none of the difficulties usually described. If a diverging result is obtained in a series of determinations the possibility of organic contamination may be checked up by ashing the titration fluid and reestimating the calcium. In the absence of contamination identical results will be obtained. In this way we have also shown that no contamination of the oxalate occurs if ordinary precautions are observed (Table II).

The advantage of deproteinization over ashing methods in the analysis of blood for inorganic elements lies in the great saving of time where numerous determinations must be carried out. Prior to its use by us for calcium and magnesium this principle had been used by Greenwald (4) in the determination of acid-soluble phosphorus and had been shown by McLean and Van Slyke in the case of chlorides (5) to be applicable where a high degree of accuracy is desired.

Picric acid is a satisfactory protein precipitant, is readily purified, can be used in hot solution containing mineral acid, and being less soluble in the presence of acid is in great part removed before the precipitation of the calcium. Our data show that calcium is readily and completely precipitated in the presence of the amounts of picric acid found in our filtrates. High concentrations slow the reaction, so that in running a blank on this reagent it should be ashed.

The precipitation of the calcium is based on McCrudden's procedure. The ammonium oxalate is added to keep the concentration of oxalate ions high. It is safer to let stand over night for precipitation.

In Table I are given the results of a series of determinations by the permanganate titration method upon pure solutions containing besides calcium and magnesium relatively large amounts of phosphates and iron. In the Solution I the amounts of these interfering constituents are far greater than those tested by McCrudden as given in his original papers, and inasmuch as tissues anywhere near as high as this in iron are much lower in phosphorus the conditions of precipitation are much more difficult than those we would expect to meet with in the analysis of the ash of any ordinary biological material.

The results show that calcium may be accurately determined under these extreme conditions. They cannot be taken as representing the attainable accuracy, but inasmuch as no particular effort was made to adapt the determination to the widely varying amounts of calcium present, the results illustrate the flexibility naturally associated with a titration method.

In Table II are given the results of a series of determinations made upon serum, plasma, whole blood, cerebrospinal fluid, human milk, and human semen. In some cases the protein precipitate was ashed and calcium determined as a second check upon the uniformity of the calcium distribution. This point was also checked by determinations on serum to which known amounts of calcium were added. Direct checks on the purity of the calcium oxalate precipitates obtained from the various biological fluids are given in Nos. 36 to 43. These are of course also checks upon the whole precipitation and titration procedures. That the results obtained by deproteinization are in

general slightly higher than those by ashing, can, in view of the direct checks on calcium distribution, only be due to the tendency of the latter to run a trifle low. While figures are given for calcium in 100 cc. of serum, etc., it must be borne in mind that the actual determinations represent from 0.3 to 1.0 mg. of calcium.

The four determinations upon human semen besides illustrating the applicability of the method are of interest as revealing the relatively high calcium content of the fluid in question, this varying apparently with the content of the spermatozoa high in nuclein.

With ordinary care and using the amounts indicated in the methods results within 2 per cent of the true values may be regularly obtained. The method possesses no fixed limitations as to

TABLE I.

Estimation of Small Amounts of Calcium in the Presence of Relatively Large Amounts of Magnesium, Iron, and Phosphates.

No.		Solution taken.	Ca	
			Found.	Theory.
		cc.	mg.	mg.
1	Solution I (in 100 cc.: Ca 7.28 mg., MgO 10 mg., Fe ₂ O ₃ 50 mg., P ₂ O ₅ 100 mg.).	100	7.30	7.28
2	" "	100	7.29	7.28
3	" "	100	7.25	7.28
4	" "	25	1.83	1.82
5	" "	25	1.81	1.82
6	" "	25	1.83	1.82
7	" "	10	0.728	0.728
8	" "	10	0.733	0.728
9	Solution II (in 100 cc.: Ca 7.17 mg., MgO 5.0 mg., Fe ₂ O ₃ 2.0 mg., P ₂ O ₅ 20 mg.).	100	7.16	7.17
10	" "	100	7.18	7.17
11	" "	25	1.79	1.79
12	" "	25	1.82	1.79
13	" "	7	0.507	0.502
14	" "	7	0.507	0.502
15	" "	4	0.290	0.287
16	" "	4	0.292	0.287
17	" "	2	0.143	0.143
18	" "	2	0.145	0.143

TABLE II.
The Determination of Calcium in Blood Serum and Other Biological Fluids.

No.		Ca per 100 cc.	
		By ashing.	By deproteinization.
		mg.	mg.
1	Ox serum No. 8.	10.16	10.22
2	" " " 8.	10.08	10.01
3	" " " 10.	10.47	10.48
4	" " " 10.	10.50	10.57
5	" " " 10.		10.52
6	" " " 10.		10.30
7	" " " 10. Calcium determined in both filtrate and precipitate.		10.46
8	" " "		10.47
9	Ox serum No. 10. 0.05 mg. Ca added per cc.		15.37
10	" " " 10. 0.05 " " " "		15.23
11	Human serum (eclampsia).	8.47	8.52
12	" " "		8.66
13	Ox plasma.	8.80	8.87
14	" "	8.80	8.95
15	" blood, whole, No. 10.	6.40	6.53
16	" " " 10.	6.30	6.56
17	" " " 10. Calcium determined in both filtrate and precipitate.		6.54
18	" " "		6.62
19	Cerebrospinal fluid, human.	5.13	5.03
20	" " "	5.01	5.00
21	" " "	5.04	4.99
22	Human milk No. 9.	27.63	28.00
23	" " " 9.	28.16	28.28
24	" " " 10.	32.02	32.89
25	" " " 10.	32.70	33.00
26	" " " 11.	20.65	
27	" " " 11.	20.67	
28	" " " 12.	32.61	
29	" " " 12.	32.19	
30	" " " 13.	35.10	
31	" " " 13.	35.30	
32	" semen No. 1, Subject A. Ca 3.02 per cent of ash.	20.60*	

*Mg. per 100 gm.

TABLE II—*Concluded.*

No.		Ca per 100 cc.	
		By ashing.	By deproteinization.
		mg.	mg.
33	Human semen No. 2, Subject A. Ca 2.36 per cent of ash.	20.60	
34	" " " 3. " " 2.60 " " "	23.40	
35	Human semen No. 4, Subject B.	84.50	
36	Whole blood No. 8. Titration fluid ashed and calcium reestimated.	6.40	6.40
37	" " 7. "	6.01	6.05
38	" " 9. "	9.80	9.87
39	Serum " 11. "	10.00	10.10
40	Plasma " 10. "	8.15	8.30
41	Human milk " 2. "	28.28	28.45
42	Cerebrospinal fluid No. 3. "	6.05	6.10
43	" " " 4. "	5.25	5.34

accuracy, but this depends on well known factors, primarily upon the care exercised and the amounts of calcium determined. Hence the principles and technique of these methods are adaptable to widely varying needs.

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THE CALCIUM CONTENT OF THE BLOOD SERUM IN CERTAIN PATHOLOGICAL CONDITIONS.

By JOHN O. HALVERSON, HENRY K. MOHLER, AND OLAF
BERGEIM.

*(From the Laboratory of Physiological Chemistry of Jefferson Medical
College, Philadelphia.)*

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In the tables which follow data are presented with regard to the calcium content of human blood serum in certain pathological conditions, particularly nephritis, as well as of the blood serum of normal men. The blood corpuscles are very low in calcium as has been shown by Abderhalden and others, so that any significant changes in blood calcium values will be shown by plasma values. It might appear that determinations on plasma would be preferable to those on serum but we have found changes in plasma values to be closely reflected in serum values even in uremia, where the calcium content of the blood is most markedly altered. Serum has therefore been employed as a matter of convenience.

Lyman (1) has recently presented data with regard to the calcium content of the whole blood in a considerable number of pathological conditions. This author is unable to account for the marked variations in blood calcium values obtained by him, for while the statement is made that the calcium varies but little in the great majority of subjects, values ranging from 5.2 to 9.6 mg. per 100 cc. are given for apparently normal individuals. Average figures of 6.1 for males and 7.1 for females are obtained, which the author considers a sufficiently great difference to stimulate further investigation. In another case with hemoglobin 160 per cent of standard a value of 2.8 mg. of calcium was found in the blood.

That these differences do not necessarily indicate any significant alteration in calcium metabolism but may be largely accounted for by variations in the ratio of plasma to corpuscles is apparent when we bear in mind the low calcium content of the corpuscles and the extreme variations which the ratio may undergo. The volume of plasma per unit of whole blood has also been shown to be on the average about 10 to 20 per cent greater for women than for men, though of course not constant for the

sexes. The very high and low values obtained by Lyman are thus most readily accounted for as representing respectively anhydremia and anemia or chlorosis. This view is confirmed by the low finding in dysentery with which is associated withdrawal of water from the blood, as well as by high values in cases where hemorrhage might be expected. That considerable percentage variations in blood count or hematocrit readings may occur among individuals considered normal, and that these are influenced by such physiological conditions as physical exercise, perspiration, fright, partial asphyxiation, age, the ingestion of food, changes in climate or altitude, etc., is well known.

In uremia decreases in plasma calcium and hematocrit readings frequently occur. The normal values obtained by Lyman in nephritis and other conditions might thus represent a compensation of these two factors. In view of these difficulties which are not met with in the case of plasma or serum, the determination of calcium in whole blood in disease loses most of its significance unless hematocrit determinations are also carried out, in which case the error of the latter method (an approximate one) is thrown upon the calcium determination.

The present authors have already presented data with regard to the calcium content of the blood in various stages of pulmonary tuberculosis (2). The methods used were those previously described by two of us (3). In Table I figures are given for the calcium content of human serum in a variety of conditions. For normal men values from 9.6 to 10.8 mg. with an average of 10.2 were obtained. That these values are fairly characteristic is supported by the finding of similar values in the great majority of pathological cases not known to be associated with disturbances in calcium metabolism. Thus a similar range was found for tuberculosis and may be noted for most of the cases here tabulated. This is true even of Cases 22 and 23 (Table I) where the clotting power of the blood was markedly disturbed. On the other hand an undoubtedly low value was obtained in one of two cases of eclampsia. A decrease in calcium was also noted in hematogenous jaundice. Less marked decreases were found in other forms of jaundice studied and in a case of pneumonia.

In Table II are presented data from a number of cases of nephritis of different types and degrees of severity, as well as cases of edema without nephritis, and of pernicious vomiting of pregnancy. In most cases urinary analyses were carried out in the attempt to establish some relationship between the acid, ammonia, and particularly calcium excretion in the urine and the

level of plasma calcium. Total acidity, hydrogen ion concentration, and ammonia were determined by usual methods and the urinary calcium by the micro method previously described (3), albumin being first removed.

TABLE I.
Calcium Content of Human Blood Serum in Certain Pathological Conditions.

No.	Sex.		Ca (mg. per 100 cc. of serum).
1	M.	Normal.	9.6
2	"	"	9.9
3	"	"	9.9
4	"	"	10.1
5	"	"	10.3
6	"	"	10.3
7	"	"	10.5
8	"	"	10.8
9	"	Syphilis, Wassermann positive + 4.	9.4
10	"	" " " + 4.	9.7
11	"	" " " + 3.	10.8
12	"	" " " + 2.	10.3
13	"	Jaundice, hematogenous; serum cholesterol low.	7.8
14	F.	" severe; serum cholesterol high.	9.2
15	"	" catarrhal; cholesterol normal.	9.2
16	"	" marked; with pancreatitis.	9.4
17	"	Eclampsia.	8.5
18	"	" 15 days after parturition; daily urinary Ca excretion, 0.03 gm.; ammonia 0.5 gm.	9.6
19	M.	Osteoarthritis, chronic. Ca whole blood, 6.8.	10.4
20	"	Osteomyelitis, chronic.	9.7
21	"	Hodgkins' disease.	10.8
22	F.	Gall bladder disease; very slow clot formation.	10.5
23	M.	Leukemia; no organized clot formation; daily urinary Ca excretion, 0.015 gm.	9.8
24	"	Pneumonia, lobar; low cholesterol in serum.	8.7
25	"	Diabetes; plasma Ca, 10.2, urine Ca normal.	10.1
26	F.	Acute anterior poliomyelitis; 6 years of age.	9.3
27	M.	Tuberculosis of the kidney.	10.3
28	"	" pulmonary.	10.6
29	"	Sciatica.	9.6
30	F.	Constipation, chronic.	9.8

TABLE II.
Calcium Content of the Serum in Nephritis.

No.	Sex.		Serum.	Urine (daily excretion).				
			Ca (mg. per 100 cc.).	Volume.	Total acidity.	pH.	Ammonia.	Ca.
				cc.			gm.	mg.
1	M.	Nephritis, chronic, mild; plasma Ca, 9.5.	9.4	420	124	5.90	0.08	8
2	"	Nephritis, chronic, mild; plasma Ca, 9.1.	9.4	1,750	Alk.		0.40	90
3	"	Nephritis, chronic, mild; plasma Ca, 9.2.	9.5	1,625	195	6.80	0.46	31
4	"	Nephritis, chronic; plasma Ca, 9.3.	9.4	2,040	482	6.91	1.12	220
5	"	" cardiorenal.	9.7					
6	"	" acute, severe.	10.2					
7	"	" " "	9.7					
8	F.	" cardiorenal.	8.9					
9	M.	" uremic whole blood Ca, 5.9.						
10	F.	" chronic	9.2					
		8 days later, no improvement.	9.3					
11	"	Nephritis, severe, acute.	7.1					
12	M.	Uremia, comatose.	6.8					
13	"	" plasma Ca, 6.9.	7.1	980	129	6.70	0.33	8
14	"	Nephritis, severe, acute; plasma Ca, 7.6.	7.8	780	312	6.80	0.52	6
		4 weeks later, improved.	8.6	1,300	281	6.70	0.39	18
		After 2 days calcium lactate (5 gm. per day).	9.0	1,400	282	6.80	0.38	20
15	F.	Uremia, comatose; non-protein N, 47 mg.	8.1	460	178	6.21	0.00	9
		3 days later, after 1 day calcium lactate (5 gm. per day); improved.	8.7	240		6.30	0.17	6
		After 2 days' treatment.		750	268	6.30	0.32	44
		" 3 " "	9.2	455	200	6.10	0.72	20
		2 days after treatment ceased.		201	67	6.00	0.14	13
16	"	Uremia, comatose; plasma Ca, 8.0; some urine lost.	8.2	150	86	6.50	0.16	20
		5 days later, after 1 day's treatment (5 gm. Ca lactate).		325	88	6.60	0.44	43
		After 2 day's treatment.	10.1	131	55	6.70	0.21	15

TABLE II—*Concluded.*

No.	Sex.		Sg- rum.	Urine (daily excretion).				
				Ca (mg. per 100 cc.).	Volume.	Total acid- ity.	pH.	Ammonia.
					cc.			gm. mg.
17	M.	Nephritis, cardiorenal.			1,900	137	6.9	64
		1 day later, no treatment.		8.4	1,800	89	7.2	0.07 35
		After 1 day calcium lactate (5 gm.); marked edema.			920	74	7.2	0.69 28
		After 2 days' treatment.			350	41	7.1	0.16 10
		" 3 " "		9.6	480	111	7.1	0.44 10
		2 days after treatment ceased.			655	162	6.8	0.64 28
18	"	5 " " " "			405	191	6.4	0.26 17
		Nephritis, cardiorenal.		8.1	305	145	6.7	0.49 42
		2 days later after 1 day's treatment (5 gm. Ca lactate).			650	267	6.7	1.03 133
		After 2 days' treatment.			620	191	6.7	0.21 90
19	"	" 3 " "		7.9	400	204	6.7	0.25 53
		5 days after treatment ceased.			600	121	6.8	0.43 32
		Nephritis, cardiorenal with marked edema; urinary albumin 2.8 parts per thousand.		10.2	1,000	190	6.7	0.01 33
		1 day later after 1 day's treatment (5 gm. Ca lactate).			800	225	6.7	0.05 35
20	"	After 2 days' treatment, improved.			1,100	163	6.7	0.02 39
		" 3 " " albumin 1.7 parts per thousand.		10.4	900	122	6.7	0.01 39
		1 day later, cathartics only.			600	168	6.8	0.04 22
		2 days " no treatment.			910	290	6.6	28
		Edema due to abdominal neoplasm.			300	147	6.8	0.52 4
		1 day later.		8.9	290	102	6.8	0.56 3
21	F.	2 days later after 1 day's treatment (5 gm. Ca lactate).			435	127	6.8	0.65 5
		After 2 days' treatment.			410	123	6.7	0.48 3
		" 3 " "		9.0	550	113	7.1	0.59 5
		3 days after treatment ceased.			438	118	6.7	0.39 3
		5 " " " "			250	75	6.6	0.27 2
		Pernicious vomiting of pregnancy.		10.1	1,070	545	6.7	2.30 161
		1 day later after sodium bicarbonate administration.			460	163	6.9	1.19 14
		2 days later, able to leave hospital.			450	168	6.9	0.28 12

The most obvious conclusion from these data is that in uremia the serum calcium values are nearly always low. In this condition low values for calcium have been shown to occur by Marriott and Howland (4) who have correlated these findings with high inorganic phosphate of the serum and the existence of an acidosis which may be secondary to the increase in phosphate, the latter probably also accounting for the decrease in blood calcium. While this decrease in serum calcium is notable as contrasted with findings in most other conditions, the fact that values of less than 8.0 mg. are rare, even where the condition approaches coma, rather leads us to emphasize the great constancy of this element as compared with most blood constituents. *A priori* this might appear remarkable in view of the insolubility of the carbonates and phosphates of calcium and consequent difficulties of excretion and metabolism. On the other hand it appears that the plasma is a saturated or supersaturated solution of calcium bicarbonate. Hence any appreciable or permanent increases are not readily brought about, while at the same time the calcium present is not readily reduced in amount by precipitation, nor by excretion on account of the large reserves of calcium in the bones. Thus in nephritis notable decreases in calcium appear to occur only in the most severe types where we know the blood composition to be in other respects markedly altered. In mild or chronic nephritis or cases mainly cardiac in character the calcium values were normal or but little below normal. In uremia comatose in character values of 7.0 to 8.0 were usually obtained, the lowest value being 6.8.

The composition of the urines as far as acidity and ammonia are concerned serve mainly to emphasize the fact that the acidoses of uremia are due mainly to retention and very little to overproduction of acid. In fact in many cases the urinary acidities and ammonia outputs were subnormal rather than otherwise. This supports also the view of Marriott and Howland that the acidosis is due to acid phosphate which has but little tendency to withdraw ammonia from the tissues.

The urinary calcium excretion in nephritis, particularly in the severe types, was usually very low. Thus in Cases 13, 14, and 15 (Table II) only about 10 mg. of calcium were excreted per day as compared with average normal excretions of 100 to 300

mg. Clearly the low serum values cannot be attributed to urinary losses of calcium. It would rather appear that the low plasma calcium affects excretion unfavorably, while there appears also to be a lowered capacity of the kidney cells to excrete calcium. Inasmuch as calcium appears to be excreted by these cells as an acid phosphate the same disturbance in cellular metabolism would account for the retention of both phosphate and calcium.

The influence of calcium lactate in amounts of 5 gm. per day was studied in several cases of nephritis. In four cases of severe uremia perceptible increases toward the normal in serum calcium were noted with some simultaneous improvement in clinical condition. It must however be borne in mind that similar increases in serum calcium were found where improvement occurred without calcium administration. In two cases no appreciable increase in serum calcium was noted but in one of these the calcium level was normal to begin with. In no cases after the administration of these large amounts of calcium were abnormally high serum values obtained. This agrees with findings by us in tuberculosis.

In most cases calcium administration led to relative increases in urinary calcium excretion, although the excretions remained low, very markedly so indeed as compared with the calcium ingestion. The influence appeared greatest in cases where urinary acidities and ammonia values were highest. On the other hand in Case 19 (Table II) where the nephritis was less severe but the edema more generalized and the ammonia excretion was extremely low calcium administration had no effect upon the excretion of ammonia or of calcium itself.

Some light is thrown on these findings by the observations made on Cases 20 and 21 (Table II) which were non-nephritic in character. The first of these was a case of edema due to an abdominal neoplasm. Here the serum calcium level was but little below normal and was unaffected by calcium administration. Only 2 to 5 mg. of calcium were excreted daily by way of the urine and this excretion was entirely uninfluenced by the administration of large amounts of calcium salts.

Quite different were the findings in pernicious vomiting of pregnancy. Here although serum calcium was normal the ex-

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cretion of calcium, ammonia, and acid was very high. After alkali administration there was an immediate drop in calcium excretion to less than 10 per cent of the original value. Similar rapid decreases in acidity and ammonia were noted, and by the 4th day the patient had nearly recovered. Apparently calcium excretion increased with an excessive acid production, secondarily to the heavy drain on the alkali metal reserve which is more readily mobilized. When alkali was given the calcium excretion became at least temporarily subnormal.

SUMMARY AND CONCLUSIONS.

The calcium content of human blood serum was determined in several normal cases and in a number of pathological conditions. In the normal cases values lying between 9 and 11 mg. of calcium per 100 cc. were obtained. In nearly all of the pathological conditions studied, including cases where the blood clotted with extreme slowness, a similar range was observed, indicating a great constancy of this element in the blood serum. Distinct decreases were noted in cases of hematogenous jaundice, eclampsia, pneumonia, and particularly uremia. In several cases of uremia increases in serum calcium were noted on improvement in the clinical condition and following administration of calcium lactate. The urinary calcium excretion in severe nephritis was found to be low and calcium lactate administration brought about but slight absolute increases. Where marked general edema occurred, with or without nephritis, the excretion of calcium was unaffected by increased ingestion. In a case of pernicious vomiting of pregnancy with severe acidosis, alkali administration decreased calcium excretion to 8 per cent of its original value.

It is pointed out that calcium determinations on whole blood are of little value unless the relative volume of plasma and corpuscles is known.

The authors desire to express their obligation to Dr. Philip B. Hawk for the opportunity of carrying out this work and for suggestions.

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A BIOLOGICAL ANALYSIS OF PELLAGRA-PRODUCING DIETS.

II. THE MINIMUM REQUIREMENTS OF THE TWO UNIDENTIFIED DIETARY FACTORS FOR MAINTENANCE AS CONTRASTED WITH GROWTH.

By E. V. McCOLLUM AND N. SIMMONDS.

(From the Laboratory of Agricultural Chemistry of the University of Wisconsin, Madison.)

(Received for publication, September 14, 1917.)

In a former paper from this laboratory McCollum and Pitz (1) have discussed the etiology of experimental scurvy in the guinea pig and its bearing on the "vitamine" hypothesis of Funk, which postulates the existence of a number of unidentified dietary essentials each of which acts as a protective substance. According to this hypothesis the absence of one or another of these leads to the development of a specific syndrome; in one case scurvy, in another beri-beri, in another rickets, and in still another pellagra. McCollum and Pitz demonstrated that scurvy in the guinea pig could not possibly be a "deficiency disease" in the sense in which Funk and his school employed this term. In other papers McCollum, Simmonds, and Pitz (2) have made it clear that the several symptom complexes recognized as being due to faulty diet can easily be accounted for by unsatisfactory relationships among the well recognized constituents of the normal diet. We have therefore, in the light of a great amount of systematic study of simplified diets, been forced to accept the view that among the diseases enumerated the "vitamine" hypothesis is a satisfactory explanation for polyneuritis only, and that of the four this is the only "deficiency disease" in the sense intended by Funk.

That another "deficiency disease" is possible and sometimes occurs there can be little doubt. McCollum and Davis (3)

first pointed out that certain mixtures of fats of animal origin, as butter fat, egg fat, and the fats extracted from the internal organs, *e.g.*, the kidney, liver (4), etc., contain something which is absolutely indispensable for either maintenance or growth, and that this substance is not found in vegetable oils or fats and in but very small and inadequate amounts in the body fats of animals. It is present in abundance in the leaves of plants and in a very few seeds, conspicuous among the latter being flaxseed and millet seed. We have shown how when the diet is inadequate in its content of this substance which we designate fat-soluble A, the animals become emaciated and suffer from edema of the eyes. Blindness results if the animals are permitted to go without this dietary essential or with an inadequate supply for a sufficient time. We have many times rescued animals from the threshold of death by the administration of one of these growth-promoting fats when nutrition had failed because the diet contained fats from vegetable sources only and no other source of the fat-soluble A.

In 1904 Mori (5) in Japan first called attention to what he believed to be fat starvation in infants which he observed in times of famine, and described eye troubles suggestive of those which we have found to result from this specific dietary deficiency. He pointed out the efficiency of chicken liver and of eel fat as curative agents. Mori observed over 1,400 cases in children of 2 to 5 years affected with xerosis of the conjunctiva and keratomalacia terminating often in blindness. Mori says the disease does not occur among fisher folk, but among peoples whose diets are principally of vegetable origin. This is significant in determining its cause. Bloch (6) has recently described severe cases of necrosis of the cornea with ulceration in forty cases at Copenhagen. The children had been fed nearly fat-free separator skim milk, and were atrophic or dystrophic and anemic. Bloch attributed the disorder to "fat starvation," since the children responded to breast milk feeding, or, in the case of older ones, to whole milk mixtures and to cod liver oil administration. Czerny and Keller (7) describe a similar condition of the eyes in children suffering from malnutrition as the result of being restricted to a cereal diet.

We feel confident that these cases of xerophthalmia reported by Mori and Bloch should be looked upon as a "deficiency disease" not hitherto recognized in its true relation to diet. It is not, as these authors believe, a "fat starvation" which produces the condition, but a lack of the unidentified dietary factor, fat-soluble A, which occurs in just those foodstuffs which they observed to possess curative properties. Since we are in possession of a method for producing an experimental xerophthalmia, we plan in the immediate future a thorough study of this condition.

There are then, we believe, two deficiency diseases in the sense in which Funk employed this term. One of these is polyneuritis which Funk recognized. This is due to a lack of an adequate supply of the unidentified water-soluble B which is abundant in all natural foodstuffs but is practically absent from purified starch, polished rice, sugars, and all fats from both animal and vegetable origin. The other we believe is the syndrome described above in which the two most prominent features are emaciation and xerophthalmia. Scurvy, pellagra, and rickets we believe can be accounted for by errors in the diet without assuming a protective substance for each of them.

Pellagra we shall discuss from the etiological standpoint in a forthcoming paper.

We have considered the diseases of dietary origin in the present paper only to present the main reasons why we hold the view that there are in reality two and only two unidentified dietary essentials in addition to the well recognized factors, proteins, carbohydrates, fats, and inorganic salts. We have secured chemical evidence which lends strong support to the idea that what we term water-soluble B is in reality but a single substance rather than a mixture containing several physiologically indispensable complexes. This phase of the problem we shall discuss under a separate title in the immediate future.

The object which we had in view in collecting the data presented in this paper will be briefly explained. It is well known that an animal during the growing period may be fed a diet adequate in all respects except that it contains too small an amount of protein of the quality contained in the food to support growth, yet enough to maintain it without loss of body weight over a long

period (8). The same idea may be expressed in the possibility of suspending growth because of a shortage of a single amino-acid in the diet. The work of Osborne and Mendel (9) gave the impression to several investigators that they (Osborne and Mendel) had discovered in lysine, an amino-acid which was necessary for growth but unnecessary for the processes of repair of tissue wasted in endogenous metabolism. The evidence rested upon experiments in which gliadin supplied 93 per cent and "protein-free milk" 7 per cent of the nitrogen of the diet and successful maintenance, but inability to grow was demonstrated. Gliadin was at that time believed to be free from lysine, hence the above conclusion. Osborne, Van Slyke, Leavenworth, and Vinograd (10) have since shown that the most carefully purified gliadin yields about 0.75 per cent of lysine, so the premise for the entire line of reasoning regarding the peculiar rôle of this amino-acid became untenable.

Sweet, Corson-White, and Saxon (11) at once attempted to make use of this supposedly established principle to control the growth of tumors. If the diet of Osborne and Mendel did not support growth, would a neoplasm, which is analogous in many respects to embryonic tissue be able to grow upon a host receiving such a food supply? While their results seemed to substantiate the view that a tumor is not able to grow so well on a host restricted to the experimental diet, the data lose somewhat in interest since the theory on which the work was done has been found to be based upon erroneous reasoning.

The protein is evidently not the most satisfactory dietary factor upon which to attempt to differentiate between the conditions essential for maintenance as contrasted with growth. Van Slyke and Meyer (12) have shown that even during a prolonged fast the normal content of amino-acids is maintained in the blood and muscles through autolysis of the muscle proteins. This fact alone would make it improbable that such an experiment as that of Sweet, Corson-White, and Saxon should succeed. Van Slyke and Meyer's studies revealed a factor of safety of the greatest importance in the mechanism whereby during starvation the blood always contains a suitable pabulum to support the nutrition of the nervous system, internal organs, and heart even at the expense of great wastage of the skeletal muscles.

Another line of attack of the problem of controlling tumor growth has recently been tested by Benedict and Rahe (13) through such an adjustment of the diet as would insure maintenance of the adult tissues of the host, but make growth impossible. They sought such an adjustment in limiting the experimental animals to a diet adequate in all respects except for its content of "vitamine." The latter they supplied by small additions of yeast to the food. These investigators hoped to determine whether the normal adult tissues or the tumor tissue would be able to appropriate the supply of "vitamine" when the latter is present in the food in amount just sufficient to support life without loss of body weight. Their results indicate that the tumor cell can under these circumstances draw a supply of the water-soluble B from the blood stream even under conditions which require retrogressive changes in the normal body tissues.

Our charts give the results of a series of experiments designed to show the magnitude of the interval between the requirements of young rats for each of the unidentified dietary factors, fat-soluble A and water-soluble B, for maintenance as contrasted with growth. For comparison a series of tests with full-grown rats is described which, it was hoped, would give a clue to whether the adult tissues could maintain themselves on a supply of one or both of these dietary essentials lower than that required by the young for maintenance or growth.

We selected wheat germ as a source of the water-soluble B and employed butter fat as the source of the fat-soluble A. The diet employed was known to be satisfactory for normal growth and prolonged maintenance of health when an adequate supply of both of the unidentified essentials was furnished. *It consisted of casein 18.0, dextrin 76.3, salt mixture (185) 3.7, and agar-agar 2.0, (Chart 1). This was fed with (a) an abundance of fat-soluble A (as butter fat), (b) of water-soluble B (in wheat germ), and (c) with varying amounts, but always below the optimum requirements, of both wheat germ and butter fat as carriers of these unknown substances.*

These experiments it was hoped would give an answer to several questions of physiological interest.

1. Assuming that each of the food products, butter fat and wheat germ, are of constant quality what is the lowest intake of

each which can supply enough of the A or B respectively to just prevent loss of weight? This assumes, as has been previously shown and is illustrated anew by Chart 1, that an abundance of either unidentified factor without the other will not prevent rapid decline.

With our purified food mixture combined with wheat germ 1 per cent and butter fat 1 per cent, one adult rat has lived more than 13 months. It was not satisfactorily nourished on this diet, and for want of enough of the unknown A and B nearly all rats confined to such a ration may be expected to die within a few months (Chart 2, Lot 662). In this case the shortage of the factor A was greater than for the B. Our results indicate that there is no low plane of intake of either of these substances which can be said to maintain an animal without loss of vitality. When the minimal amount necessary for the prevention of loss of weight is approached the life of the animal is jeopardized if the diet is persisted in.

2. When both essentials A and B are supplied in amounts just above the maintenance needs, will growth be proportional to the supply of these, or must a certain excess of each over this minimum be present before growth in the young can take place?

Charts 4, 5, and 6 indicate that within certain limits growth is proportional to the supply of the fat-soluble A and water-soluble B in the diet, all other factors being properly adjusted. Failure of some to grow where others do so indicates that, even with our stock of animals which has been carefully selected for years by elimination of unfit adults from the breeding stock and the selection of the finest specimens for breeding, uniformity of performance under nutritive conditions adverse in these specific ways is not to be expected.

3. Is the requirement of either A or B for maintenance or growth less when the other of the two unknowns is present in liberal amounts, than when both are supplied in amounts near the minimum?

The answer to this question is definite and clearly demonstrated by our observations in many experiments in which two or more dietary factors fall below the optimum in quality or quantity. We have previously described rations derived from wheat germ with purified food additions, with which for a few

weeks normal growth may be attained, followed later by depression of growth with permanent stunting; or in certain cases full adult size may be attained but failure partial or complete in reproduction or rearing of the young has been observed. The improvement of a single dietary factor, which may be protein, inorganic content, or fat-soluble A, can enable animals on these same diets to perform their functions in a nearly normal manner (14). A quantity of either the fat-soluble A or the water-soluble B which may be just sufficient when all other dietary factors are of satisfactory quality, will not, when a second factor is less well constituted, induce well-being in the same degree as when a more generous supply is furnished. The animal can tolerate being limited to a very low intake of either the dietary A or B much better with an otherwise excellent diet than when it is less well constituted. (Compare Lots 663 and 665, Chart 3.)

4. What is the effect on the health of animals, of limiting them to a minimum supply of either the dietary A or B the remaining one being supplied in abundance; or of limiting the supply of both to near the minimal requirements? The answer to this question we expected to obtain by observing whether as the actual minimum for each of the two substances was approached the mortality of the animals should greatly increase while a few were able to maintain body weight and the appearance of health over a long period.

Without exception the records shown in the charts accompanying this paper indicate that it is a dangerous procedure to attempt to fast an individual selectively for one or both of these dietary essentials. We believe therefore that efforts directed toward the control of tumor growth by this means can never become of practical value. The life of the host will be cut short if the experimental conditions imposed are sufficiently rigid to render growth impossible.

The death of the animals, whose curves are marked P, was preceded by loss of muscular control. From the similarity of the behavior of these rats and of pigeons which are brought into a pathological state from a diet of polished rice, we believe that the rats exhibit the typical symptoms of polyneuritis.

Credit is due to Mr. W. Pitz for assistance in the preparation of materials employed in this work.

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CHARTS.

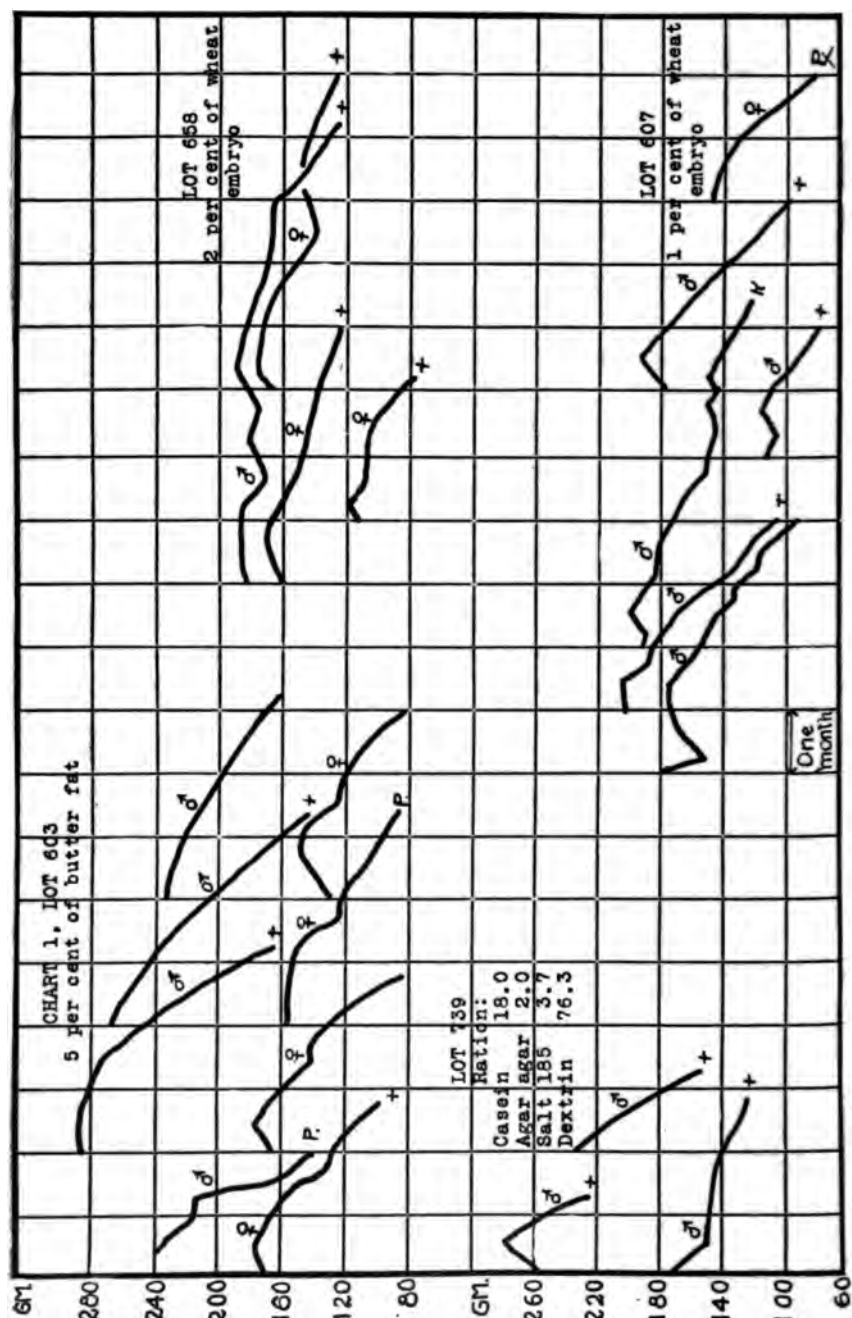


CHART 1. Lot 739. These curves illustrate the rate of decline in body weight of adult rats fed a diet which was adequate in protein, carbohydrate, and inorganic salts, but lacking both the unidentified dietary factors, fat-soluble A and water-soluble B.

Lot 603 received the same food as Lot 739 but with 5 per cent of butter fat to furnish the fat-soluble A. Without the second unknown essential factor B, loss of weight is uninterrupted and death follows in from 60 to 100 days. 5 per cent of butter fat furnishes an abundance of the dietary A, as is shown by many instances in our records of animals which have grown from weaning time to normal maturity and brought forth and reared the normal number of young (four or five litters).

Lots 607 and 658 show that in adult rats about 1 year old and in good condition and capable under favorable conditions of living 2 years longer, the span of life is greatly shortened by limiting the animals to the content of unidentified dietary factors contained in 1 or 2 per cent of wheat germ. With 1 per cent of germ loss of weight was fairly rapid while with 2 per cent two died after 2 and 4 months, two others lived 5 and 7 months respectively and death supervened with but little loss of weight.

Wheat germ contains a small amount of the fat-soluble A (15) and a liberal amount of the water-soluble B. When supplemented with a suitable amount of butter fat (fat-soluble A) 2 per cent of wheat germ furnished enough of the factor B to support growth (Chart 5, Lot 838).

These records are of particular importance for the light which they throw upon the debilitating effects of greatly restricting the intake of either or both of the two unidentified dietary essentials. The prospects for controlling tumor growth through reducing the content of either of these substances in the diet are not favorable for such reductions are attended by great danger to the host.

Composition of salt mixture 185:

	<i>per cent</i>
NaCl.....	0.173
MgSO ₄ (anhydrous).....	0.266
NaH ₂ PO ₄ ·H ₂ O.....	0.347
K ₂ HPO ₄	0.954
CaH ₄ (PO ₄) ₂ ·H ₂ O.....	0.540
Fe citrate.....	0.118
Ca lactate.....	1.300

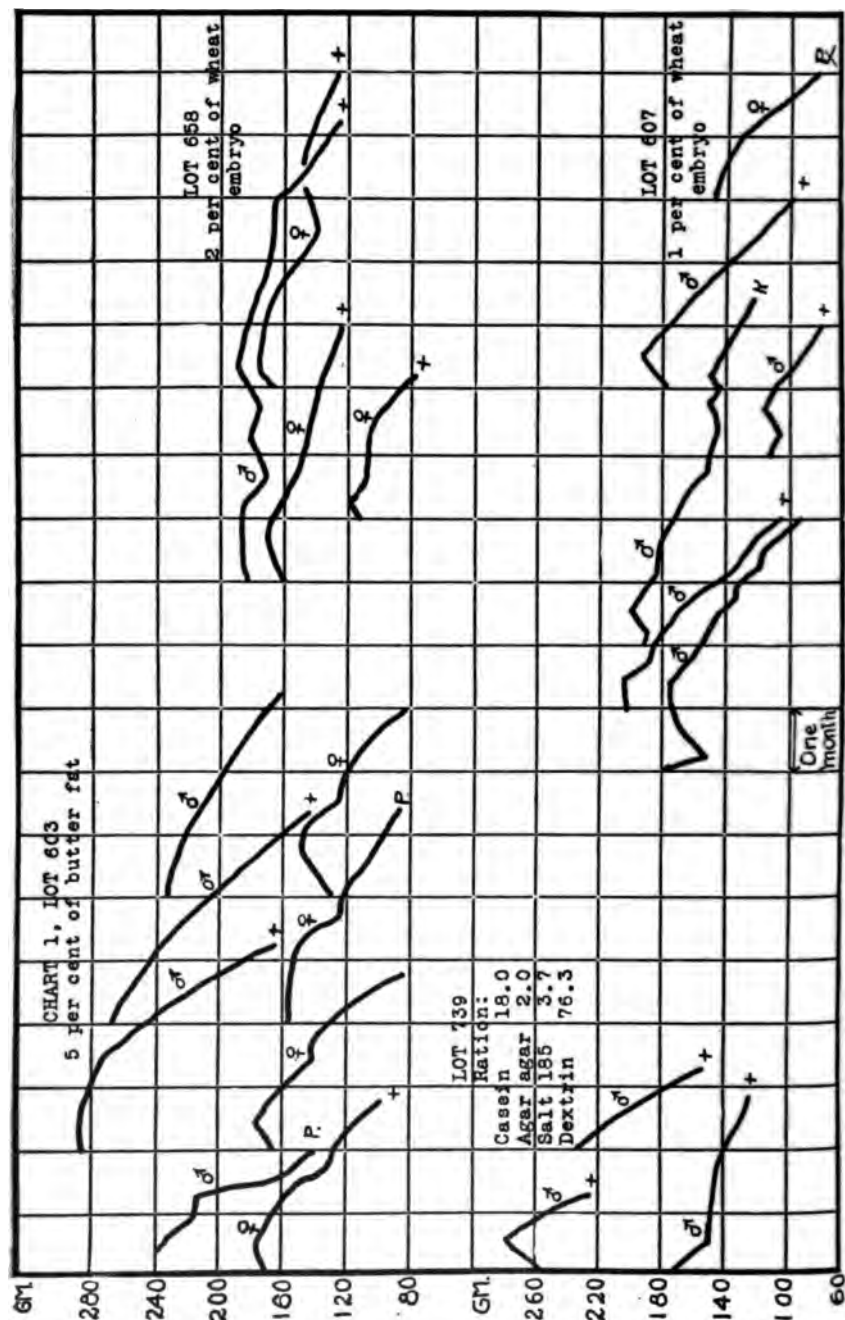


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Fe citrate.....	0.118
Ca lactate.....	1.300

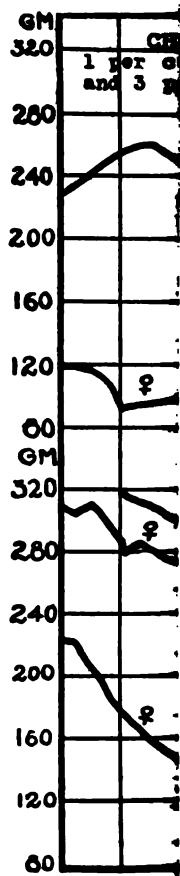
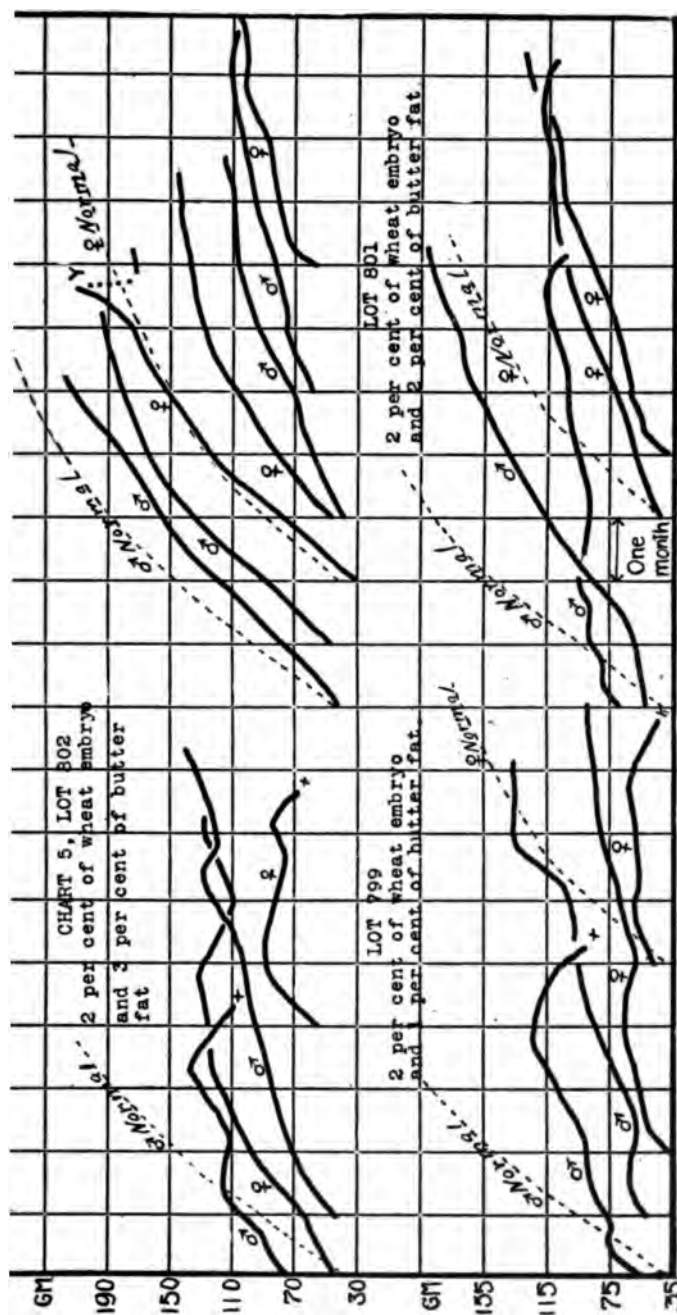


CHART 2. 1
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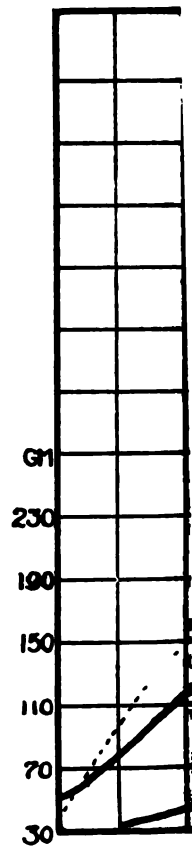
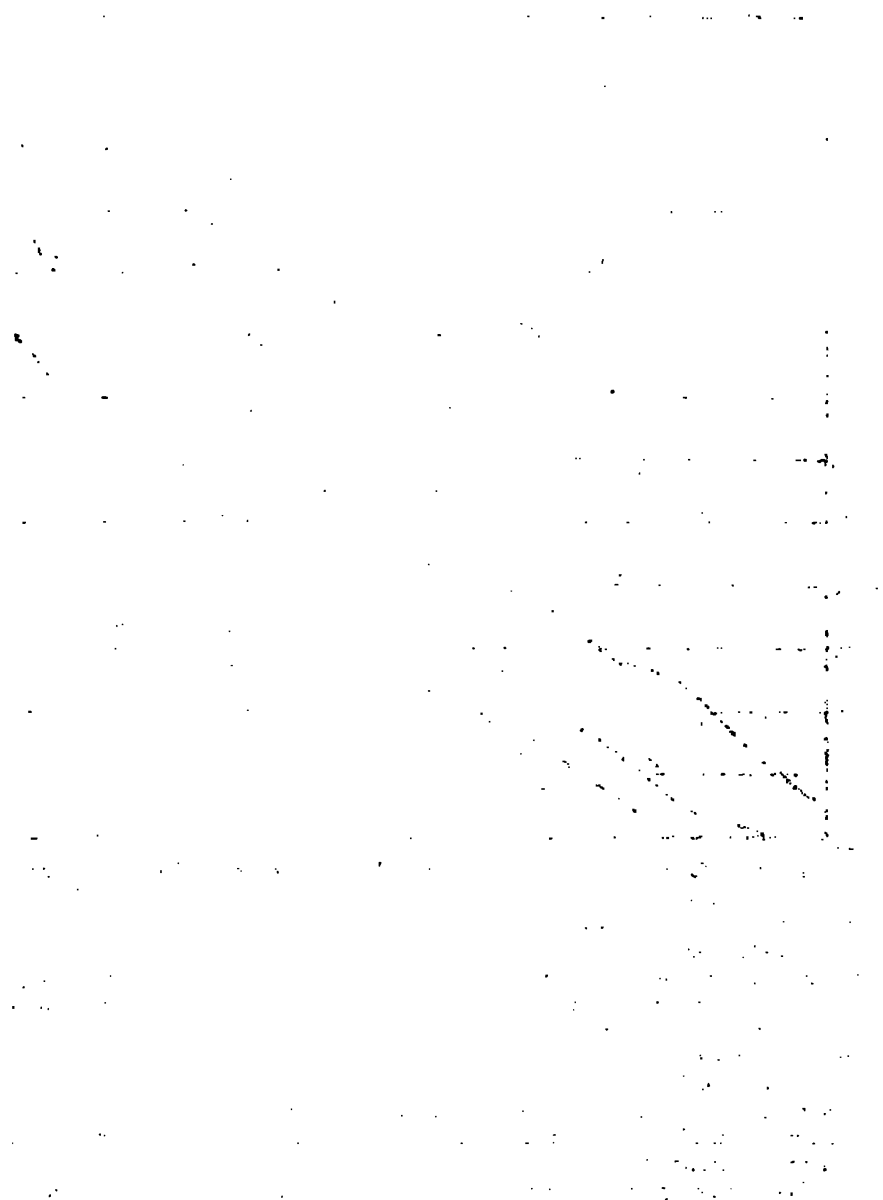


CHART 6. L
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THE PROBABLE ACCURACY, IN WHOLE BLOOD AND PLASMA, OF COLORIMETRIC DETERMINATIONS OF CREATININE AND CREATINE.

By ANDREW HUNTER AND WALTER R. CAMPBELL

(From the Department of Pathological Chemistry, University of Toronto.)

(Received for publication, August 29, 1917.)

Certain investigations, of which a preliminary account has already been published,¹ have required us to carry out within the last two years a large number of determinations of creatinine and creatine not only in whole blood, but also in plasma. The methods employed—those described by Folin,² and in slightly modified form by Myers and Fine³—were at first assumed to be in each of these situations equally and entirely reliable; but it soon became obvious to us that no final answer to the questions engaging our attention was possible until this assumption had been adequately tested. We were accordingly led to consider rather closely what degree of precision was actually attributable to the various determinations we were making.

Our first attempt⁴ to reach a decision upon this point led us to the conclusion that, if the disturbing influence of the sodium picrate color be eliminated by the use, in the interpretation of the colorimeter readings, of empirically constructed standard curves, and if care be taken to protect the picric acid solutions employed from the deteriorating action of light, the determination of minute quantities of creatinine may be accomplished with a highly satisfactory degree of accuracy. Essentially the same conclusion was reached by Folin and Doisy,⁵ who indicated a second method of minimizing the picrate effect, and demonstrated the frequent presence in fresh commercial picric acid of a chromogenic impurity similar to, if not identical with, the one which we had encountered only in old solutions. One positive result of these investigations was the vindication of the Folin method for creatinine against the severe strictures passed upon it by McCrudden and Sargent.⁶ Unfortunately, as far as the

¹ Hunter, A., and Campbell, W. R., *J. Biol. Chem.*, 1917, xxix, p. xviii.

² Folin, O., *J. Biol. Chem.*, 1914, xvii, 475.

³ Myers, V. C., and Fine, M. S., *Chemical Composition of the Blood in Health and Disease*, Cooperstown, N. Y., 1915, p. 19.

⁴ Hunter and Campbell, *J. Biol. Chem.*, 1916-17, xxviii, 335.

⁵ Folin, O., and Doisy, E. A., *J. Biol. Chem.*, 1916-17, xxviii, 349.

⁶ McCrudden, F. H., and Sargent, C. S., *J. Biol. Chem.*, 1916, xxiv, 423.

positive demonstration of that method's accuracy was concerned, neither of them went further than the case of pure aqueous solutions. They did not suffice to prove the entire reliability of the determination in such a complex mixture as the blood. The reaction upon which the method is based is not a specific one; and we possess no guarantee that the blood does not normally contain, besides creatinine, other substances capable singly or collectively of simulating its behavior. Uncertainty upon this point affects not only the determination of preformed creatinine, but even more strongly that of creatine; for in the latter case one has to reckon with the possible development, as products of hydrolysis, of quite new reacting substances. It is a further complication that the case of whole blood in relation to these determinations is not necessarily the same as that of plasma; and there are occasions when it is important to know, if not the absolute, at least the relative accuracy with which creatinine or creatine can be determined in these different situations.

Considerations such as these imperatively suggested the need of proceeding beyond the case of pure solutions, and of attempting to ascertain as closely as possible the limitations in actual practice of every application of the colorimetric method for creatinine to blood analysis. The need was emphasized, as we proceeded, by the criticisms which from time to time have been directed against one or another of these applications by other workers in the field. Thus it has been maintained by Gettler⁷ that the Folin method for the determination of blood creatinine, although capable on suitable modification of giving trustworthy results, is in its original form grossly inaccurate. More recently Wilson and Plass,⁸ while conceding the correctness of that method as applied to plasma, have held that it exaggerates somewhat the true creatinine content of whole blood; and have expressed similar opinions regarding the relative value, in blood and plasma, of the Folin method for creatine. A lack of confidence in the latter is to be inferred also from a preliminary communication by Greenwald.⁹ In our own examination of the subject we have of course found it necessary to take into account the views of each of the authors mentioned.

A decisive test of the accuracy of the colorimetric procedure, in any of its applications, might be furnished by the discovery of a really specific reaction, or by the quantitative isolation of the creatinine, preformed or total, in an identifiable combination. It is at present, and will probably long remain, impossible to check the method in any such manner. We have been forced, therefore, in endeavoring to reach a reasoned decision upon the

⁷ Gettler, A. O., *J. Biol. Chem.*, 1917, xxix, 47.

⁸ Wilson, D. W., and Plass, E. D., *J. Biol. Chem.*, 1917, xxix, 413. Also Plass, E. D., *Bull. Johns Hopkins Hosp.*, 1917, xxviii, 137.

⁹ Greenwald, I., *Proc. Soc. Exp. Biol. and Med.*, 1916-17, xiv, 115.

question, to rely upon evidence less direct in character. Such evidence we believe ourselves now to possess in sufficient abundance to warrant certain fairly definite conclusions. It has been obtained by the application of a variety of tests, which were based upon the following considerations.

1. When sodium hydroxide is added to pure creatinine in a saturated solution of picric acid, the reduction of the latter does not take place instantaneously but with a certain measurable velocity. If the amount of the colored reduction product be plotted against the time, the progress of the reaction will be graphically illustrated by a curve. This curve will have a characteristic form, and each time the Jaffé reaction is performed with pure creatinine under standard conditions the same type of curve will be reproduced. If the typical form be departed from, it can only mean that some other reaction, with a different velocity constant, is sharing in the development of the color.

2. Provided that colorimeter readings are interpreted, according to our practice, by reference to standard curves,⁴ dilution of a pure creatinine solution will result in a strictly proportionate diminution of its colorimetrically estimated concentration; in other words, the final outcome of the determination will be independent of the dilution at which it is made. This is a simple consequence of the manner in which the standard curve is constructed. A mixture of creatinine with another similarly reacting substance would not necessarily obey this rule; for it might happen to exhibit a somewhat different relation between concentration and color intensity. If the determination, as made at one dilution, should fail to agree with that made at another, one would be justified in concluding that creatinine was not the only chromogenic substance present.

3. If a pure creatinine solution be compared with each of two standards, one of higher and one of lower concentration, the two determinations will give (within the ordinary limits of colorimetric work) identical results. This, like (2), follows from the structure of our interpretative curves. If the relation which insures this agreement be disturbed, and results be found to vary according to the standard employed, it will be evidence that at least a part of the total color owes its origin to something other than creatinine.

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4. Applying these principles to the case of blood, we see that, if the color reaction there is due to creatinine alone, it must develop at such a rate as to yield a time curve identical with that of pure creatinine in the indicated concentration; the result of the determination must be independent of the extent to which the blood is diluted; and it must be a matter of indifference what particular concentration of creatinine is adopted as a convenient standard. The fulfillment of all these conditions will furnish not indeed absolute proof, but at least a strong presumption, that we are dealing with an unmodified creatinine reaction; the failure of any one of them will be conclusive evidence to the contrary.

From the points of view thus indicated we have examined the creatinine reaction as developed in picric acid filtrates from whole blood and plasma both before and after autoclaving. The methods for preformed and total creatinine were thus subjected to three distinct series of tests, the results of which will now be severally discussed.

The Rate of Color Development.

In order to study the rate of color development in creatinine solutions or blood filtrates the following procedure was adopted. A suitable standard¹⁰ is placed in position in one of the colorimeter cups, and a watch indicating seconds is laid beside the instrument. The alkali is added to a conveniently large volume of the solution to be tested, and the time at which a complete mixture is effected is noted to within a couple of seconds. The solution is immediately placed in the second cup of the colorimeter, and the plunger is sunk until its side of the field is distinctly the lighter. The plunger is then carefully raised until the two sides just appear to have attained equality. The time is instantly noted, and the reading is taken at leisure. Again the plunger is depressed, and the whole proceeding repeated. In this way it is possible to make a succession of quite deliberate readings at the rate of at least one per minute. After 10 minutes

¹⁰ In all the experiments of this section the fixed standard employed was one containing 0.2 mg. of creatinine in 100 cc. of saturated picric acid—a "1 mg." standard.

these readings are interrupted, but additional ones are taken at 15, 25, 40, and sometimes 60 minutes after the addition of the alkali. The creatinine values of the readings, ascertained by reference to our standard curves,⁴ are plotted on squared paper against the times at which they were taken; and finally a smoothed curve is drawn through the points thus charted.

Crude as this method may appear, it is surprising how successfully, under favorable conditions, it can be operated, and how rarely any point is found to lie at more than a negligible distance from an ideal curve. Occasional irregularities were of course encountered, but never in such frequency or of such magnitude as to throw doubt upon the substantial fidelity of our curves to the actual course of each reaction.

Experiments have been made, and curves constructed, for each of the following cases:

1. A "1 mg. standard" of pure creatinine.
2. A "1.5 mg. standard" of pure creatinine.
3. A "2.5 mg. standard" of pure creatinine.
4. A standard corresponding to 1 mg. of creatinine with 100 mg. of glucose per 100 cc.
5. The filtrate from whole blood as obtained by the original Folin technique, in which saturated picric acid solution is added directly to the oxalated, but otherwise untreated blood.
6. The same filtrate after autoclaving.
7. The filtrate from whole blood treated according to the technique of Myers,⁵ in which the blood is laked before being saturated with picric acid.
8. The filtrate from picric acid treatment of blood plasma.
9. The same after autoclaving.

The total number of curves constructed was forty-two, the minimum for any given case being two, and the maximum eleven. To tabulate the many separate observations from which these were derived, or even to reproduce individually all of the curves themselves, would be to take up a great deal of space without displaying very clearly their significance. The special characteristics of each group of curves can be more easily and more perspicuously illustrated by combining its members into a single composite curve. To effect this, all the curves of one category were first projected together upon the same chart, quantities of

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creatinine being plotted as ordinates, time intervals as abscissæ. It was then noted at what points each of the abscissæ representing successive whole minutes cut the various curves. These points were translated into mg. of creatinine and averaged. The averages for every minute up to 10, and for the subsequent 15, 25, and 40 minute periods, were finally plotted upon a fresh chart, and joined to form a continuous curve.

The advantages of this procedure are obvious. It not only simplifies the presentation of our results, but nullifies, even more completely than is possible on the original curves, the influence of any accidental irregularity in the observations. The only possible objection to it is that it might impart to the results in any one group the appearance of a uniformity which they did not in reality possess. It deserves therefore to be expressly emphasized that no one of the original curves was incompatible with the conclusions drawn from the composites. The differences exhibited by the latter were found also in every single series of the former. Sometimes they were less striking, just as often more so; they always existed, and had always the same character. We are confident, therefore, that our method of presentation involves nothing more than the averaging of entirely consistent sets of results, and that, when the number of separate curves combined is large enough, the composite may be presumed to give a very accurate representation of the typical course of the reaction studied.

The curves themselves are shown on Charts I and II. The final averages from which they are drawn are recorded in Table I. The interpretation of the curves is facilitated by the calculation from these averages of the expression

$$k = \frac{1}{t} \log_e \frac{a}{a-x}$$

which is the velocity constant of the reaction on the assumption that it is a monomolecular one. The values found for k are likewise exhibited in Table I. It should be noted that they are calculated with reference to the first 10 minutes only, during which alone creatinine can be held responsible for developing the color.

TABLE I.

Average Velocity of Color Reaction in Different Cases.

 x = apparent creatinine content at t minutes. a = " " " " " " " "

$$k = \frac{1}{t} \log_e \frac{a}{a-x}$$

t	Pure creatinine.						Creatinine + glucose.	Plasma.			Whole blood.				Plasma auto- claved.			Whole blood autoclaved.										
	1 mg.			1.5 mg.				2.5 mg.			Folin.		Myers.		x	a - x												
	x	a - x	k	x	a - x	k		x	a - x	k	x	a - x	k	x		a - x	k											
min.	1	0.38	0.62	0.48	0.56	0.94	0.47	0.92	1.58	0.46	0.54	0.49	0.74	0.53	0.70	0.94	0.63	0.91	1.13	0.71	0.95	1.65	0.63	1.29	1.85	0.97	1.07	
	2	0.62	0.38	0.48	0.93	0.57	0.48	1.51	0.99	0.46	0.75	0.28	0.65	0.71	0.35	0.55	1.11	0.46	0.62	1.29	0.55	0.60	1.88	0.40	0.87	2.17	0.65	0.74
	3	0.76	0.24	0.48	1.10	0.40	0.44	1.86	0.64	0.45	0.87	0.16	0.62	0.82	0.24	0.50	1.24	0.33	0.52	1.38	0.46	0.46	2.01	0.27	0.71	2.37	0.45	0.61
	4	0.84	0.16	0.46	1.23	0.27	0.43	2.10	0.40	0.46	0.93	0.10	0.58	0.90	0.16	0.47	1.32	0.25	0.46	1.45	0.39	0.39	2.08	0.20	0.61	2.53	0.29	0.57
	5	0.90	0.10	0.46	1.32	0.18	0.42	2.22	0.28	0.44	0.96	0.07	0.54	0.95	0.11	0.46	1.40	0.17	0.45	1.52	0.32	0.35	2.14	0.14	0.56	2.64	0.18	0.55
	6	0.94	0.06	0.47	1.39	0.11	0.44	2.32	0.18	0.44	0.98	0.05	0.50	0.99	0.07	0.46	1.45	0.12	0.43	1.59	0.25	0.33	2.19	0.09	0.54	2.72	0.10	0.56
	7	0.97	0.03	0.50	1.45	0.05	0.48	2.38	0.12	0.43	1.01	0.02	0.56	1.02	0.04	0.44	1.49	0.08	0.43	1.66	0.18	0.33	2.23	0.05	0.55	2.77	0.05	0.58
	8	0.99	0.01	0.58	1.48	0.02	0.53	2.43	0.07	0.45	1.02	0.01	0.58	1.04	0.02	0.49	1.51	0.06	0.41	1.72	0.12	0.34	2.25	0.03	0.54	2.80	0.02	0.62
	9	1.00			1.49	0.01	0.56	2.47	0.03	0.49	1.02	0.01	0.52	1.05	0.01	0.52	1.54	0.03	0.44	1.78	0.06	0.38	2.27	0.01	0.60	2.82		
	10	1.00			1.50			2.50			1.03			1.06			1.57			1.84			2.28			2.82		
	15	1.00			1.50			2.50			1.05			1.09			1.67			2.19			2.33			2.85		
	25	1.00			1.50			2.50			1.08			1.13			1.77			2.44			2.37			2.85		
	40	1.00			1.50			2.50			1.11			1.17			1.84			2.80			2.44			2.88		

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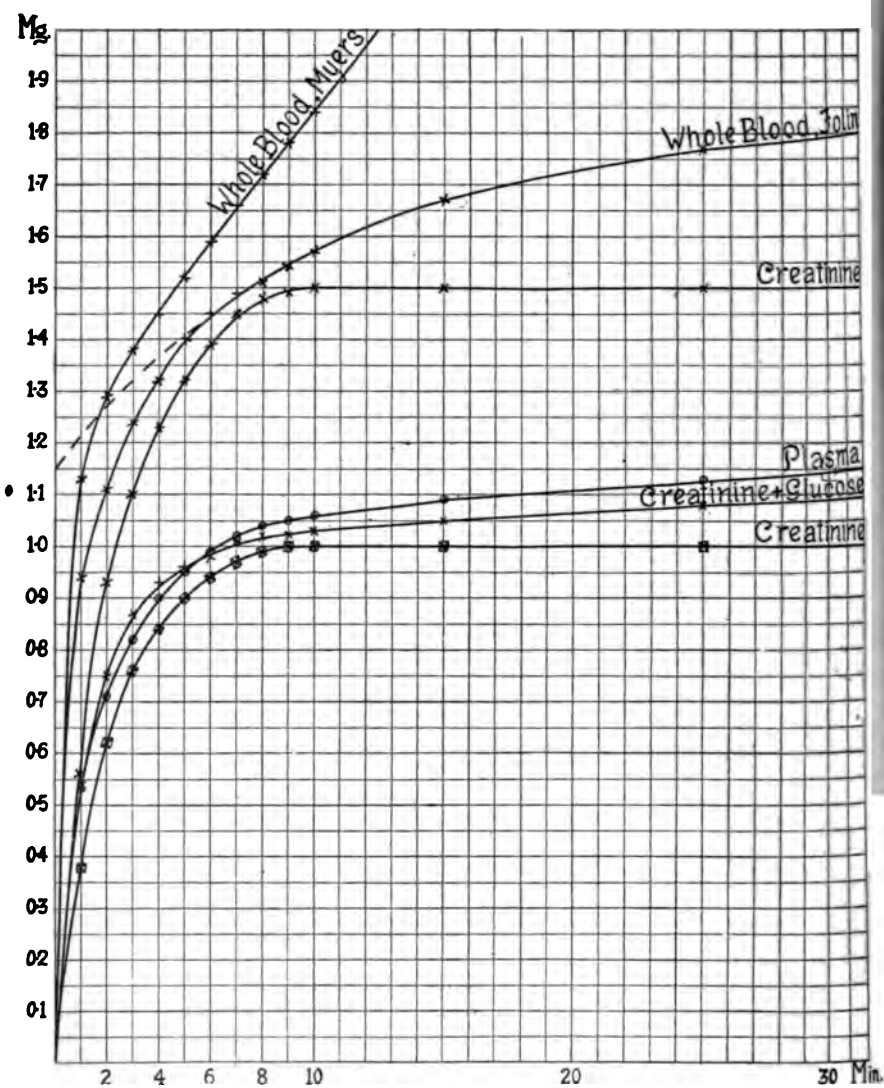


CHART I. Time curves for the reaction with creatinine, creatinine + glucose, plasma, whole blood (Folin's technique), and whole blood (Myers' technique).

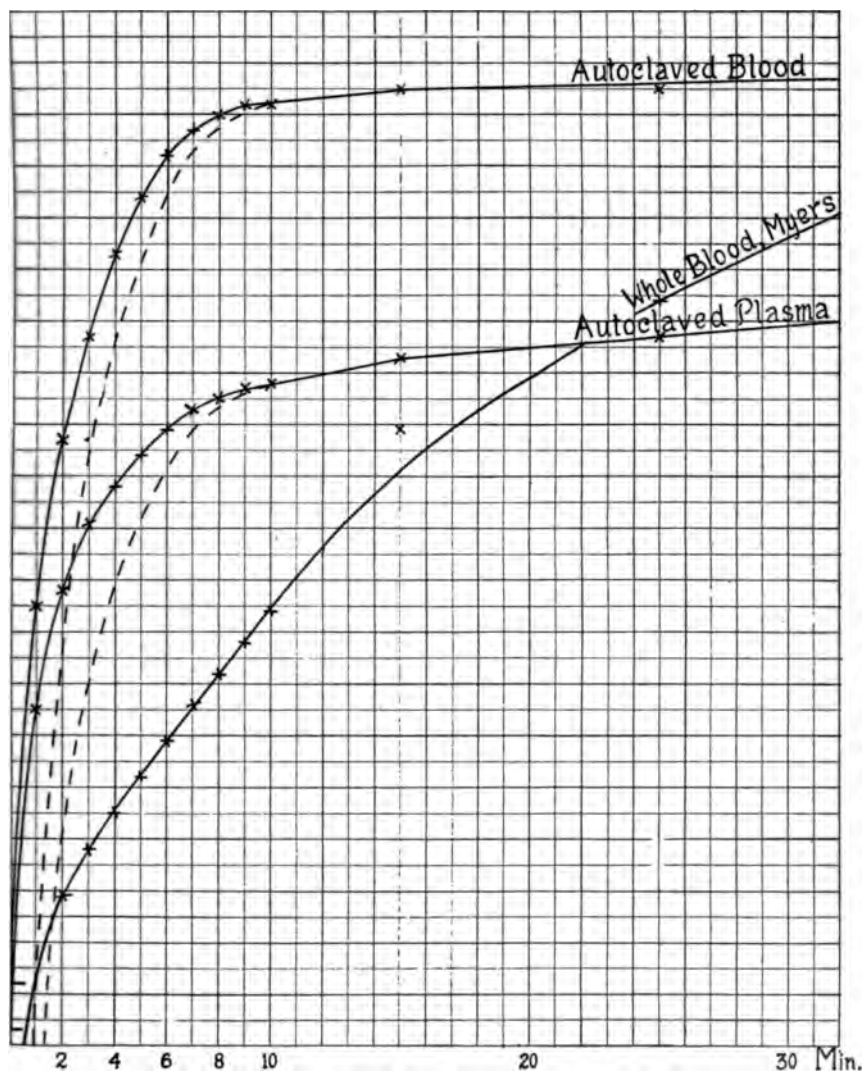


CHART II. Time curves for the reaction with autoclaved whole blood and autoclaved plasma; and continuation of the curve for whole blood (Myers' technique).

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Discussion of the Curves.

A reference to the charts will show that the eight curves finally available for comparison do not all assume exactly the same form. A discussion of each in turn will serve to elucidate the consequences which, we believe, may be drawn from the differences presented.

Pure Creatinine.—The time curve shown in Chart I for the "1 mg." pure creatinine standard is a composite constructed from three single ones, which as a matter of fact almost exactly coincided with one another. Its form indicates that at any given instant the rate of increase of the color is proportional to the amount of creatinine which has still to react. This is made clear by the fact that the value of k as recorded in Table I is as nearly constant as the nature of the method used could lead one to expect. The only aberrant value is at the end of the curve; and this is a point where a comparatively small error of observation would have a large effect in the formula by which k is calculated. The evidence therefore bears out, better even than might have been anticipated, the assumption that the reaction is a monomolecular one. For all practical purposes the reaction may be regarded as complete, and the curve as having reached its summit, at the 9th minute after the addition of the alkali. After that point the curve becomes a horizontal line.

The conclusion that we are dealing with a reaction of the first order is confirmed by the curves for the 1.5 and 2.5 mg. standards, of which the former only is reproduced in the chart. These also conform closely to the logarithmic formula, reach their maxima about the 9th minute, and yield for k a practically constant value, approximately the same as with the first curve. Rejecting as certainly erroneous all values for k higher than 0.50, the averages at the three different concentrations are 0.48, 0.45, and 0.45. The true velocity constant of the creatinine reaction is probably not far from the grand average of 0.46. It may be pointed out that with the character of the reaction thus firmly established, it becomes permissible to calculate, on the basis of the recorded observations, the time curve for any given concentration of creatinine.

Creatinine and Glucose.—The reduction of picric acid by glu-

ucose in hot alkaline solution is a familiar reaction, which forms the basis of a well known quantitative method for the estimation of sugar in blood. Gettler⁷ has recently drawn attention to the fact that the same reaction takes place, although much more slowly, at ordinary temperatures; and he appears to regard it as one factor at least in that gross exaggeration of the true creatinine content of blood to which, as he claims, the original technique of Folin conducts. He is also, it would seem, inclined to make it responsible for the progressive deepening of color which is noticeable in blood creatinine determinations long after the expiration of the prescribed 10 minute interval. That it must have some effect in each of these directions is self-evident; but the curve exhibited in Chart I, a composite from four single ones, would hardly suggest that that effect is a considerable one. At the 10 minute point it is seen that glucose, in such concentration as might be encountered in the analysis of normal blood, has contributed to the total color no more, on the average, than the equivalent of 0.03 mg. of creatinine; and even after 40 minutes it has raised the apparent creatinine content of the mixture hardly more than 10 per cent. We believe that, as a matter of fact, Gettler's reported experiments with glucose (see his Experiment II, page 52) tend to give an exaggerated idea of the probable effect of that substance in normal blood. The mixtures with which these experiments were made contain, if we understand correctly the figures of his table, not such a concentration of glucose as might be expected in a normal blood filtrate, but twice as much.¹¹ Nevertheless, in view of the striking character of the results reported, we have thought it worth while to control the evidence of our time curve by some additional experiments. These consisted simply in ascertaining, by comparison with appropriate standards, the amount of color developed on adding the prescribed proportion of alkali¹² to various combinations of glucose and creatinine in saturated picric acid solu-

¹¹ For 6 mg. in 15 cc. of blood filtrate would be 6 mg. in 3 cc. of blood, or 2 per cent.

¹² It should perhaps be expressly stated that in all our work we have used the proportion of sodium hydroxide recommended by Folin (0.5 cc. of 10 per cent solution for 10 cc.). Gettler employs a greater concentration, namely, 1 cc. of 2 N NaOH to 10 cc.

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tion. The combinations tested and the results obtained are shown in Table II.

TABLE II.
The Effect of Glucose on the Creatinine Determination.

Experiment.	Creatinine per 100 cc.*	Glucose per cc.*	Standard of comparison at 20 mm.	Colorimeter readings.			Apparent creatinine content.	
				10 min.	1 hr.	24 hrs.	10 min.	1 hr.
	mg.	mg.		mm.	mm.	mm.	mg.	mg.
1	—	1.0	Sodium picrate.	18.3	16.7			
2	—	2.0	" "	17.3	15.5			
3	—	3.0	" "	16.2	13.8			
4	—	4.0	" "	14.4	12.8			
5	1.0	—	1 mg. creatinine.	20.0	20.1	19.9	1.00	0.99
6	—	1.0	1 " "	50	42**	{ 19.1 10.7		
7	1.0	1.0	1 " "	20.0	18.5	{ 15.5 7.7	1.00	1.12
8	1.0	1.0	1 " "	19.7	17.9		1.02	1.18
9	1.0	1.0	1 " "	19.5	18.5		1.04	1.12
10	1.0	1.0	1 " "	19.6			1.03	
11	1.0	4.0	1 " "	17.5	15.1	1.0	1.22	1.52
12	1.0	4.0	1 " "	18.2			1.15	
13	0.4	—	1 " "	34.0			0.42	
14	0.4	4.0	1 " "	27.0	20.8	0.9	0.62	0.94
15	0.4	4.0	1 " "	27.0			0.62	

* The figures in these columns are not the actual concentrations, but those to which they would correspond in blood analysis, namely, five times as much.

** At 4 hours.

The first four experiments of this table were designed to ascertain what intensity of color may be produced within short periods of time by glucose alone. For this purpose a creatinine standard was inconveniently dark, and the one used was a simple picric acid solution treated with the regular amount of alkali. Bearing in mind the very light tint of such a standard it will be seen that the glucose of a normal blood (Experiment 1) could yield very little color in an hour, and no more than a practically negligible quantity within 10 minutes. Elsewhere⁴ we have shown that the color of the sodium picrate standard is about two and a half times less intense than that of a "1 mg." creatinine standard. It may therefore be calculated that on the

scale of the latter the glucose color at 10 minutes in Experiment 1 would be equivalent to $(20 - 18.3) \times \frac{2}{3} = 0.7$ mm., which corresponds to something like 0.05 mg. of creatinine. This estimate is confirmed by Experiments 7 to 10, in which the maximum effect, upon the 1 mg. standard, of a glucose concentration comparable to that of normal blood, is seen to be only 0.5 mm., corresponding to 0.04 mg. of creatinine.¹³ A greater effect becomes of course noticeable in time. Within an hour it would correspond in a normal blood to about 0.14 mg. of creatinine (average of Experiments 7 to 9), in 24 hours to 1 to 2 mg. (Experiments 6 and 7).

Quantities of glucose greater than normal react with considerably greater rapidity. According to Experiments 11 to 15 a concentration equivalent to 0.4 gm. per 100 cc. of blood may in 10 minutes yield enough color to simulate 0.15 to 0.22 mg. of creatinine; the apparent increment may amount in 1 hour to 0.5 mg.; and after 24 hours the solution will be so dark as to be almost opaque. It is clear therefore that the glucose factor may assume considerable importance whenever there is a notable hyperglycemia, and it is quite possible that some of the creatinine values reported in cases of diabetes and of nephritis may on this account have been slightly exaggerated. But our results with normal proportions of sugar make it exceedingly doubtful that these could, within 10 minutes, produce any effect of consequence, or that the addition, as recommended by Gettler, of equivalent amounts of glucose to the standard is a prerequisite of a successful creatinine determination.

A point which is not directly important in the present connection, but which may be mentioned parenthetically, is that the glucose color is less permanent than the creatinine one, and that side by side with its gradual development there takes place, presumably as the result of oxidation, a gradual fading. The amount of glucose color present at any given period depends therefore on circumstances which are not always entirely controllable. This is illustrated in Experiments 6 and 7, where two different readings are reported for the 24 hour period. Of these

¹³ The colorimeter readings of the table, like all which we may have occasion to report, are interpreted by reference to a standard curve constructed by and for the individual observer.

the lower one in each case was given by a portion of the mixture which had been kept in a tall narrow test-tube, the higher by one which had been exposed in an Erlenmeyer flask to a greater surface of air. This susceptibility to reoxidation (if that be the correct explanation) may account for the fact that although in actual determinations of blood or plasma creatinine the color is usually deeper at 24 hours than at 10 minutes, the extent of its increase is very variable and not infrequently it is actually lighter.

To return to the time curve, the indications of which are merely confirmed by the other experiments reported, it will be seen that the glucose effect, negligible as it may be in practice, does not leave the form of the curve altogether unaffected. Indeed, to judge from the glucose experiments, the method of time curves is rather more delicate than we had expected it to prove. The expression k , as calculated from the data for the first 10 minutes, is no longer a constant, being distinctly higher at first than it afterwards becomes. The glucose component causes the curve to begin its rise a little more steeply than the pure creatinine one; it abolishes the maximum at 9 minutes, although at that point the completion of the creatinine reaction is not entirely concealed; and it induces a subsequent gradual and prolonged rise, proceeding apparently at a constant rate so long as the observations were continued.

Although all four of the curves, which were combined into the one shown, exhibited these characters with greater or less distinctness, they did not coincide with one another so entirely as the pure creatinine ones. This slight variability is in accord with the additional results reported in Table II. It is not pronounced enough to influence materially any conclusion we have drawn from the composite curve.

Plasma.—The plasma curve shown in Chart I represents the average of eleven experiments on as many individuals. As it happens, the average creatinine content of these, assuming the correctness of the Folin method, was not far from 1 mg. (1.06 mg.) per 100 cc. This greatly facilitates direct comparison of our plasma curve with those for creatinine and for creatinine with glucose. It will be seen that it assumes a form intermediate between the two. Like the latter it rises rather steeply at

first, yielding in the first few minutes higher values for k than in the later ones. Like the latter also it exhibits after the 9 minute point a continued rise, during which indeed the resemblance increases to an almost absolute parallelism. But its deviation from the logarithmic formula with a constant k of 0.46, although unmistakable, is distinctly less marked than that of the curve with glucose. In fact, from the 3rd or 4th minute to the 9th the plasma curve becomes indistinguishable from a pure creatinine one. It is clear that the differences existing between plasma and pure creatinine solutions are more than accounted for by the glucose content of the former; and it is difficult to resist the conclusion that the determination of creatinine in a plasma filtrate is likely to be free of serious error. This conclusion, we shall see, is confirmed by the other tests which we have applied.

Whole Blood (Folin Procedure).—The eleven experiments which contributed to the construction of this curve (Chart I) were carried out on the same specimens that furnished the eleven plasmas of the preceding one. The time curves for whole blood and plasma are therefore directly comparable with one another. Since, moreover, the average apparent creatinine content of the group of whole bloods was 1.57 mg. per 100 cc., the time curve for the 1.5 mg. standard furnishes another convenient basis of comparison.

In accordance with data already reported by us in a preliminary communication,¹ and since then supplemented by many others not yet published, the curve for whole blood lies at a considerably higher level than that for plasma. It assumes, at the same time, a decidedly different form. In every respect it departs yet further from the monomolecular formula. It begins its rise even more steeply, shows an even greater variability of k , passes the 9 minute point with an even less conspicuous change of direction, and continues with an even more decided upward trend thereafter. The contrast between the two curves is perhaps most obvious to the eye in the interval from the 9th to the 15th minutes, in which the apparent creatinine content of the blood increases 0.13 mg., that of the plasma only 0.04.

Since at the 9th minute the creatinine reaction is complete,

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the color developing subsequently, as well as that which distorts the earlier part of the curve, must be due to something else. This something else cannot be merely glucose, for blood contains no more glucose than plasma, and the curve for the latter, as well as that for creatinine with glucose, has already given us the measure of the glucose effect. The obvious deduction is that we are dealing here with some unknown substance, present in the corpuscles only, reacting slowly as compared with creatinine, but rapidly enough to produce an appreciable result within the time prescribed for the colorimetric comparison. This deduction may not be entirely sound; it is at least conceivable that the effect is a glucose effect after all, but one which has been catalytically accelerated by something of corpuscular origin. The point is immaterial. What matters at present is the result, and that is clearly such as to render the colorimetric determination of creatinine in whole blood subject to a decided error of overestimation.

The probable magnitude of this error can be roughly gauged from the form of the curve. Between the 9th and 10th minutes the unknown substance contributes color enough to counterfeited 0.03 mg. of creatinine. In the preceding 9 minutes there must therefore have developed the equivalent of at least 0.27 mg. This would reduce the content of actual creatinine from 1.54 (the apparent amount at 9 minutes) to 1.27. Even this estimate is still a maximum. The unknown reaction, there is every reason to suppose, proceeds with greatest rapidity during the first few minutes. Its time curve, displayed without complication by creatinine between the 9th minute and the 40th, will be found on analysis to be a logarithmic one (with a velocity constant of about 0.094) which, when projected backwards, as shown by the dotted line on the chart, cuts the zero abscissa as low as 1.15 mg. This, then, is probably the true value of the creatinine component of the combined curve. If it be accepted as such, the value found by the Folin procedure (1.57 mg., reading at 10 minutes) would be an overestimate of 36.5 per cent. The apparent excess of creatinine possessed by whole blood in comparison with plasma is by this conclusion very sensibly diminished.

Upon the quantitative details of the foregoing argument we.

do not desire to lay more stress than the derivation of the curve would warrant. It is not pretended that the rather minute analysis attempted has the force of a mathematical demonstration. Our claim is merely that it proves the existence of a not entirely negligible disturbing factor, and enables us to form a probable estimate of its average effect.

The difference between the reactions with whole blood and with plasma was illustrated not only by the composite curves reproduced but also by every one of the eleven pairs of individual curves constructed. Especially obvious in each case was the lack of parallelism between the 9th and 15th minutes. This was not always so pronounced as in Chart I, but on the other hand it was often very much more so. It would appear therefore that the error of the whole blood determination may be in different specimens greater or less than the average estimate which we have reached. Seldom, if ever, can it be entirely absent.

This conclusion is fortified, as we shall see, by the results of the other tests employed; and it receives indirect support from the yet more striking issue of the experiments with the Myers' technique.

Whole Blood (Myers' Procedure).—Wilson and Plass⁸ have recently stated that when in the determination of creatinine the blood is laked before being saturated with picric acid, the values obtained are usually higher than those yielded by the original procedure of Folin. This statement we have tested and abundantly confirmed; indeed we have not encountered any human blood to which it did not apply. In extreme cases the creatinine content of laked blood has even appeared to be more than double that of unlaked.¹⁴ Upon this phenomenon we have endeavored to throw some light by constructing time curves of the reaction for this particular case also.

The curves obtained differed strikingly from those given by unlaked blood. They differed so much that if we had utilized all of our single curves in making the composite one, the latter would have passed the limits of Chart I within the first 10 minutes. We therefore made a selection, of two curves only, which

¹⁴ We have received the impression that the blood of pregnant women is especially likely to show extreme differences between the results by the Folin technique and those by the other.

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would yield a composite free from this inconvenience. The specimens from which these selected curves were derived had an average creatinine content by the Folin procedure (1.53 mg.) not far from the average for the whole eleven. The composite can therefore, in spite of the limited number of observations upon which it is based, be fairly enough set by the side of those already discussed. Specially selected though it is, its whole course cannot be displayed upon Chart I. It is therefore repeated and completed on Chart II, which starts from a higher base line than the other. It is a curve, as will be seen, which exhibits in most respects merely an exaggeration of the characteristics of that for unlaked blood. In both the coefficient k presents initial values which are higher than the constant of the creatinine reaction, to be followed by later ones which sink below it; but with laked blood each of these effects, and especially the latter, is so much more prominent that the curve retains hardly a suggestion of the logarithmic form. Its course suggests that the cause of the higher values given by laked blood is the relatively rapid development of a secondary reaction which, adding itself to the creatinine one, continues with but slightly abated velocity long after the latter must have come to an end. The curve therefore not only illustrates admirably the statement of Wilson and Plass, but settles the question which they left in doubt, whether the deeper color yielded by laked blood is due to the "liberation of creatinine which is otherwise occluded in the voluminous precipitate, or merely the liberation of color-producing material not creatinine." Whatever the responsible substance may be, it is evidently not creatinine. One cannot help suspecting that it is the same substance which we have already encountered in unlaked blood, and that the destruction of the corpuscles has simply set it free in greater amount. This idea is at least not contradicted by the similar behavior of the coefficient k in the two cases. It is quite probable that even when picric acid solution is added directly to blood, some laking takes place; and perhaps if we could prevent it altogether the determination in whole blood might be made as accurate as that in plasma.

We have on a former occasion⁴ commended the Myers' technique as obviating, at one point at least of the determination,

er of introducing a picric acid solution which has deteriorated through exposure to light; but the facts now revealed doubt in our mind that the Folin procedure is to be relied upon, and that results obtained by the other are always too high, and often very much, too high. They will suffer from a defect even when the strictest attention is paid to the method for the color comparison; while, at such a rate does the development of the extra color continue, that the accident of a cause of error assumes greater importance than ever.

Unheated Plasma and Whole Blood.—To the color reaction in unheated blood and plasma, upon which depends the Folin estimation of creatinine in these fluids, we have devoted only a few paragraphs under the present head. This was partly because it will be shown later, the question of the accuracy of this estimation appeared to be settled decisively enough in the past, and partly because, before we reached this point in our study, it had been made the subject of published investigations by others.^{8,9} Nevertheless it seemed worth while to make a series of time curves in these cases also, if it were only to compare the results with the series, or to control the conclusions reached by other investigators.

Incidentally the attempt was rewarded by the development of some rather interesting points.

Presented therefore in Chart II composite curves for "total" creatinine, derived respectively from two whole bloods and two plasmas.

A standard of comparison for each was provided by assuming on the basis of previous observations the course which the reaction would have followed if it had been a pure creatinine estimation. The admissibility of such a procedure has already been discussed. The calculated curves are indicated by intermediate lines. It should be noted, in comparing Charts I and II, that in the latter the base line is not zero, but 1.0 mg.

Curves for unheated blood and plasma have revealed the presence of a disturbing factor peculiar to the former. This factor is probably present in autoclaved blood also. But as the estimation here is always carried out in a greater dilution of picric acid filtrate, its influence might be expected to be much less prominent. Moreover, as this factor is absent from plasma, one would be prepared to find the curve for plasma creatinine distorted only by the diminished con-

centration of sugar still present. Neither of these expectations appears at first sight to be fulfilled. On the contrary both curves exhibit a rather greater departure from the normal than any we have yet examined. There is therefore here some new element of error in operation. On a casual inspection of the curves its effect seems to be of the same kind as that already recognized. A closer scrutiny reveals a distinction. The curves now under consideration appear to spring not from the zero point of the creatinine scale, but from a higher one; from somewhere near 1.12 in the case of whole blood, and 1.03 in that of plasma. This would mean that the extra color complicating the creatinine reaction is in the present case one which develops instantaneously upon the addition of the alkali. This idea is supported by the fact (commented upon already by Wilson and Plass⁶) that the growth in depth of the color is in autoclaved blood filtrates visibly much more rapid than in untreated ones. It is supported also by dissection of the curves with the aid of the coefficient k . This assumes at first the highest values yet observed, and remains, even in its subsequent decrease, above the level of the creatinine constant. If, though, at each point of the curve there be deducted from the observed creatinine value a constant quantity of 1.12 mg. for blood and 1.03 mg. for plasma, and if corrected values for k be calculated upon the fresh basis thus arrived at, it will be found that these form in either case a series which differs less actually from the standard fixed by pure creatinine than do the figures for unheated blood or even plasma. This is made evident in Table III, where the corrected coefficients (designated, to prevent confusion, k') are exhibited alongside of the original ones, taken from Table I, for creatinine and unheated plasma respectively. It may be remarked that the corrections introduced affect, in the formula for k' , the values of a and x , but not of $a - x$.

While once more disclaiming for such calculations any exaggerated importance in a quantitative aspect, we think that they render exceedingly plausible our suggested interpretation of the curves, that, namely, they are simple creatinine ones superimposed upon an initial color instantaneously produced by some thing else. If this view of the matter be indeed correct, we can form an approximate estimate of the absolute and relative magni-

TABLE III.
Corrected Coefficients (k') for "Total" Creatinine Curves.

$$k' \text{ (blood)} = \frac{1}{t} \log_e \frac{1.70}{a-x}$$

$$k' \text{ (plasma)} = \frac{1}{t} \log_e \frac{1.25}{a-x}$$

t min.	k		k'	
	Creatinine (average).	Plasma.	Autoclaved blood.	Autoclaved plasma.
1	0.47	0.70	0.56	0.69
2	0.47	0.55	0.48	0.51
3	0.46	0.50	0.44	0.51
4	0.45	0.47	0.44	0.46
5	0.44	0.46	0.45	0.44
6	0.45	0.46	0.47	0.44
7	0.47	0.44	0.50	0.46
8	0.52	0.49	0.55	0.47
9	0.53	0.52		0.54
10				

tude of the error in creatine determinations. The whole blood filtrates, after autoclaving, had been diluted for the colorimetric comparison in the proportion of 3:10; the blood therefore contained apparently an average of 9.40 mg. of "total" creatinine; deducting from this $\frac{1}{8}$ of the assumed correction $(1.12) = 3.73$ mg. we reach a true creatinine-creatine content of 5.67 mg. As the preformed creatinine of the same bloods was found to be on the average 1.59 mg., the creatinine from creatine was 7.81 mg. according to the original observation, and 4.08 according to the corrected one. The error in the estimation of creatine amounts therefore to 91 per cent. For the plasmas the dilution was 4:5, the assumed correction 1.03 mg., and the preformed creatinine 1.08. From these data may be similarly calculated a true "total" creatinine content of 1.56 mg., a true creatine of 0.48 mg., and an observational error of 1.29 mg., or 270 per cent. Roughly, it may be concluded, the true creatine content of whole blood is likely to be about half, that of plasma about one-fourth, of the amount indicated by the Folin method. Whatever value these estimates may possess, the existence of serious errors in both cases would appear to be thoroughly established.

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Though the initial color of our hypothesis may be the main source of these errors, it can hardly be the only one. The continued increase of color which follows the main reaction indicates that the factors operating in determinations of preformed creatinine are almost certainly also still in action. In the case of whole blood they have been so diluted as to become practically negligible, for the curve after the 10th minute becomes an all but horizontal line. We have over and over again observed that the delayed color change is smaller with whole blood creatinine determinations than with any other. With plasma it seems to have become, as a result of the autoclave treatment, if anything more prominent than before; but the observations on this point were too limited in number to give much significance to this apparent inconsistency.

The Effect of Dilution upon the Determination.

The amount of preformed creatinine in plasma, or even in whole blood, is already so small that the effect of dilution upon its determination could not be conveniently tested in specimens containing only the normal proportion. In estimating the magnitude of this effect it was necessary, therefore, to rely largely upon experiments with the blood of nephritics. The fact that such blood contained without doubt an abnormal accumulation of many other substances than creatinine would, it may be supposed, render the tests only the more severe.

Apart from this detail the plan of the experiments hardly calls for description. A determination was first carried out on the usual fivefold, or in the case of total creatinine ten- or fifteenfold dilution of the blood. Thereafter the blood filtrate was further diluted with selected proportions of saturated picric acid, and other determinations were made. To avoid complicating the result with the possible influence of variables other than the concentration, the standard employed with any one set of experiments was always the same, and the dilution was never carried beyond the limits within which that standard could be suitably applied.

The results obtained in many such experiments are illustrated by the group reported in Table IV. This constitutes a complete series upon a single specimen of blood, that of a nephritic, K,

who furnished material also for some of the experiments reported in the next section. The selection of this series for reproduction is made particularly appropriate by the fact that it includes for each application of the method instances of the maximum as well as the minimum error encountered. Table IV is therefore justly representative of the whole number of experiments performed.

We have come to the conclusion, as the result of considerable experience, that the inherent error of the colorimetric procedure,

TABLE IV.

Influence of Dilution upon the Creatinine Determination.

	Dilution factor.*	Creatinine per 100 cc.**		Error.	
		Found.	Expected.		
Preformed creatinine.					
Whole blood (Folin).	1	3.76	—	—	—
	1.5	2.62	2.51	+0.11	+4.4
	2	1.86	1.88	-0.02	-1.1
Whole blood (Myers).	1	5.18	—	—	—
	1.8	2.68	2.88	-0.20	-7.0
	2	2.20	2.59	-0.39	-15.1
	2.5	1.94	2.07	-0.13	-6.3
Plasma.	1	3.85	—	—	—
	1.5	2.55	2.57	-0.02	-0.8
	2	1.85	1.92	-0.07	-3.6
Total creatinine.					
Whole blood.	2	4.40	—	—	—
	3	2.94	2.93	+0.01	+0.3
	4	2.23	2.20	+0.03	+1.4
	5	1.72	1.76	-0.04	-2.3
Plasma.	2	3.08	—	—	—
	3	2.02	2.05	-0.03	-1.5
	4	1.47	1.54	-0.07	-4.5

* 1 signifies the usual fivefold dilution of the blood.

** If multiplied by the dilution factor, gives the amount per 100 cc. of the blood concerned.

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when applied to very dilute solutions of pure creatinine, and when using standard curves to interpret the readings, may be as much as, but should not be greater than 5 per cent. If this estimate of the permissible error be accepted, it will be seen that variations in the extent to which the blood is diluted affect the determination of creatinine in one case only, that, namely, in which the corpuscles have been laked before the addition of picric acid. This emphasizes the liability to error of that particular variation of the technique. Otherwise the application of the dilution test reveals no distinction between the several cases under consideration, and fails therefore to provide any assistance in solving the main problem. The experiments performed were nevertheless not entirely fruitless, for they provide a check upon those which are next to be discussed, and add to the significance of their very different outcome.

The Influence of Different Standards upon the Determination.

In order to ascertain how far any or all of the determinations met the third criterion proposed (that a change of standard should not affect the result), we have adopted one or other of the following plans.

1. When the amount of blood filtrate available was sufficient (experiments with subjects K, Cr, S, A, and B in Table V) the comparison of results with different standards was effected by making two separate experiments; in each due care was taken to use the same amount of alkali and to make the reading at exactly 10 minutes after its addition; but while in one the standard of comparison used was "1 mg." of creatinine, in the other it was "2.5." In most instances the two experiments were performed at the same dilution, one so chosen that the actual creatinine content lay conveniently between the two standards. In some (as in Experiments 14 to 17 among those reported) the dilution was varied as well as the standard; but we have already seen that dilution, *per se*, is without significant effect.

2. If too little blood had been secured to make the above procedure possible (subjects Ca and H of Table V), the reading was first made with a 1 mg. standard; this was then as quickly as possible replaced by a 2.5 one, and a second reading taken; finally

the first standard was returned to the colorimeter, and a last reading secured. The average of the results by the first and third observations (never, of course, very far apart) was compared with that by the second. Due attention was always paid to the obvious condition, that the dilution at which the determination was made must be one appropriate for both standards.

The manner in which the various determinations, with which we are concerned, responded to one or the other of these tests is shown by the data of Table V. These are selected from ex-

TABLE V.
Effect upon the Determination of Using Different Standards.

	Subject.	Experiment No.	Creatinine per 100 cc. according to:		Error of second determination as referred to first.	Average error of group.	
			1 mg. standard.	2.5 mg. standard.			
Preformed creatinine.							
Whole blood (Folin).	Ca	1	1.79	1.95	+0.16	+8.9	+8.6
	H	2	1.72	1.91	+0.19	+11.0	
	H	3	2.02	2.24	+0.22	+10.9	
	H	4	1.80	1.96	+0.16	+8.9	
	K	5	3.60	3.72	+0.12	+3.3	
Whole blood (Myers).	K	6	4.45	4.85	+0.40	+9.0	+9.0
Plasma.	H	7	1.23	1.29	+0.06	+4.9	+1.6
	H	8	1.37	1.42	+0.05	+3.6	
	H	9	1.37	1.37	0	0	
	H	10	1.48	1.48	0	0	
	K	11	3.72	3.70	-0.02	-0.5	
Total creatinine.							
Whole blood.	K	12	8.20	8.92	+0.72	+8.8	+14.6
	K	13	7.87	8.50	+0.63	+8.0	
	Cr	14	6.19	7.11	+0.92	+14.9	
	S	15	5.57	7.00	+1.43	+25.6	
	A	16	6.60	7.65	+1.05	+15.9	
	B	17	6.35	7.25	+0.90	+14.2	
Plasma.	K	18	5.19	5.94	+0.75	+14.5	+13.6
	K	19	5.24	5.72	+0.48	+9.2	
	Ca	20	2.96	3.40	+0.44	+14.9	
	Ca	21	2.86	3.31	+0.45	+15.7	

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periments of which the total number was sufficient to give us confidence in the truly characteristic nature of the results within each group. As with Table IV, the selection is so made as to exhibit the extremes of error manifested by each of the special cases.

The results are so unequivocal that very little discussion of them is required.

With the preformed creatinine of the plasma it is clearly a matter of indifference what standard is employed. The absolutely exact agreement of some pairs of figures is of course a coincidence only. The point to be emphasized is that in none of them does the divergence exceed 5 per cent. The errors, in short, are no greater than they might be with pure creatinine solutions.

No such satisfactory accord is encountered in any of the other groups of analyses. Thus, with whole blood the outcome of the preformed creatinine determination shows an unmistakable dependence upon the choice of standard. If the 1 mg. one be assumed to give the correct result, the other yields a figure which on the average is nearly 9 per cent too high. This is an error rather too considerable to be accidental. Perhaps if it were the only evidence impugning the accuracy of the determination, one would not be prepared to consider it of decisive import; but taken in conjunction with that afforded by the time curves, it acquires indubitable significance. The fact that in one experiment (No. 5) the difference between the two results fell within the permissible range is only in appearance an inconsistency. The subject of that experiment, K, who was also the subject of all the experiments of Table IV, was one whose blood contained an abnormally high concentration of creatinine. To this the unknown agent of error bore therefore in all probability a smaller ratio than it does in normal blood, and it would be natural that it should exert a proportionally smaller influence in the analysis. Be this as it may, even the result with K does little to modify the general effect of contrast received on comparing the data for whole blood with those for plasma. The latter invariably met with success the conditions of the test; in every instance but one the former failed to.

Failure yet more decided is the outcome of every experiment

with "total" creatinine, whether in plasma or in blood. No distinction between the two cases is here apparent. Neither in one nor the other do the results with different standards ever come within reasonable range of one another. There is no escaping the conclusion that one at least of each pair of determinations must be quite considerably in error. From the figures reproduced it would appear that in the determination of creatine by the method of Folin a variation of about 1 mg. per 100 cc. with whole blood, and 0.5 mg. with plasma may depend entirely upon the choice of standard made. This is independent of the still greater intrinsic error, the existence of which was made probable by examination of the time curves.

The technique involving the laking of blood prior to saturation with picric acid had revealed such decided shortcomings in the tests already applied that we made but a single experiment with it under the present head. This was one of a series, consisting of Experiments 5, 6, 11, 13, and 19, which is perhaps of special interest in that it includes one example of each determination, all carried out upon a single specimen of blood. The blood was taken from the patient K, to whom we have already referred. The errors throughout the series are less than the averages for normal blood, a fact probably to be explained by the consideration already set forth in connection with Experiment 5; but they exhibit much the same ratios among themselves, and illustrate quite as well as do the final averages the relative position of the different cases. Among them the error of the experiment with laked blood is the highest of all. The issue serves therefore only to strengthen the unfavorable impression already formed of the Myers' technique.

The cases in which variation of the standard has been found to affect the result of the analysis are precisely those in which examination of the time curves has already revealed appreciable sources of error. The two sets of results are therefore mutually confirmatory. Their harmony extends even to the point of indicating for the several determinations the same order in relation to the magnitude of their probable error. It would appear, accordingly, very likely that the factor which makes it hard to obtain consistent results with different standards is the same as that which causes distortion of the time curve, the development,

namely, of extra color of unknown origin. To account more fully for the difficulty in relation to the standards, it would be sufficient to suppose that this extra color, whatever its source, is always of somewhat different quality from the creatinine one. This is more than a mere supposition. As a matter of fact the existence of abnormal shades of color is frequently obvious enough to the eye during the colorimetric comparison. Wilson and Plass⁸ have already drawn attention to the difficulty encountered in matching the blood color with the standard in total creatinine determinations in whole blood; in our experience the difficulty is usually (and here we have to dissent from the opinion of Wilson and Plass) just as great with plasma. A similar difficulty, not so pronounced, is sometimes encountered with the preformed creatinine of whole blood. With the preformed creatinine of plasma matching is always practically perfect. To the necessarily rather vague impressions conveyed by such observations our experiments have added precision and something at least of quantitative meaning.

Since a disagreement between results with different standards is due to something added to the creatinine, it is evident that the lower of the two estimates is always the closer to the truth. The lower result, it will be noticed, is always the one obtained with the 1 mg. standard. It follows that, while the Folin method for creatine gives results that are in any case too high, the error will be minimized by diluting the blood or plasma always so far at least as to bring the color comparison within the range of the 1 mg. standard. It is an additional argument for this recommendation that (to the eyes at any rate of both the present writers) any existing difference of color quality is less obtrusive, and the process of matching is accordingly easier, with the lower standard than with the higher.¹⁵

¹⁵ It should be stated that it is our invariable practice to set our standard, whether 1 or 2.5 mg., at 20 mm. When the stronger one is set at only 10 mm., matching of slightly dissimilar shades is apparently easier, and presumably the two standards would with such an arrangement give results that are closer together. It is perhaps not inopportune to point out further that, when the colors are not exactly alike, greater play than usual is given to the influence of the personal equation. These remarks have an obvious bearing upon the possibility of reproducing in detail our results with different standards; but they cannot affect the significance of a whole series of results by one observer using invariably the same technique.

Other Considerations Affecting the Accuracy of the Determinations.

The evidence so far considered bears solely upon the colorimetric aspects of the determination of creatinine in blood. Before it can be applied to the formation of a final judgment upon the points at issue, it is necessary to refer to certain possibilities of error in the preparatory manipulations which precede the actual colorimetry. There are two of these which have seemed to us to deserve some attention.

1. When, as the first step of the analysis, the blood is diluted, by the addition of saturated picric acid, to a volume five times greater than the original, that volume remains partly occupied by the bulky precipitate of proteins. The volume of fluid which now holds the creatinine in solution, and in which its concentration is ultimately determined, is therefore not five times the original one. The assumption that it is so will involve a not inconsiderable error of overestimation.¹⁶ The extent of this error we have attempted to ascertain by determining, with the simplified hematocrit of Epstein,¹⁷ what proportion of the total volume, after final saturation with picric acid, is occupied by the uniformly suspended precipitate. When the latter had contracted to a constant volume (which it did only after long continued centrifugation) it was found to account for, on the average, with whole blood 12 and with plasma 8 per cent of the entire volume of the mixture. Experiments with different individuals showed only inconsiderable variations from these averages. To compensate the error caused by neglecting the precipitate, it would be necessary, therefore, to deduct the above mentioned percentages from the creatinine values as ordinarily reported. The more voluminous character of the precipitate with whole blood, involving a consequently greater error in its case, is evident even in the course of the ordinary centrifugation, which forms part of the regular routine of the determination.

2. An error operating in the opposite direction is caused by the slight dilution of the blood that results from the addition of potassium oxalate solution. If the directions of Folin, to

¹⁶ Unless indeed some creatinine is adsorbed by the protein precipitate, a point on which we possess no information whatever.

¹⁷ Epstein, A. A., *J. Lab. and Clin. Med.*, 1916, i, 610.

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which we have in this respect strictly adhered, be followed, this dilution will in the case of whole blood amount to ten drops ($= 0.5 - 0.6$ cc.) in 30 cc., which is about 2 per cent. It is more difficult to estimate the exact effect on the plasma. It is of course the plasma alone which directly gains in volume when blood is diluted. The corpuscles, in consequence of the osmotic effect of the added oxalate, actually shrink. The plasma is therefore diluted not only by the water added from without, but also by fluid passing into it from the corpuscles. In order to gain some idea of the total addition to the plasma which may thus be produced we have compared in the same blood the plasma volume after (1) treatment with hirudin alone, with that after (2) the addition, as in the usual technique, of one-fiftieth volume of 20 per cent potassium oxalate solution. As the average of several such comparisons we found that a plasma volume of 57 per cent in hirudinized blood increases to 62.5 per cent in the oxalated specimen. This implies the addition to the original plasma of roughly 10 per cent of its volume. Unfortunately one cannot at once argue that the creatinine concentration of the plasma is correspondingly diminished. For it is possible, indeed likely, that the corpuscles are permeable to creatinine, and that the passage of that substance through the corpuscular envelope will ultimately compensate any effect of dilution. It is impossible to say how long it may take to bring about such a redistribution of creatinine. One can therefore hardly venture to estimate numerically the influence of the oxalate upon creatinine concentration in the plasma. The most one can say is, that it must at least partially compensate the error due to the protein precipitate, and that it may possibly neutralize it altogether. Under the least favorable assumption, that of complete and instantaneous redistribution of the creatinine, it will reduce the necessary correction from 8 per cent to 6.

With whole blood it is possible to estimate more precisely the net effect of these opposing influences. To allow for both, it will be necessary to deduct $12 - 2 = 10$ per cent from the observed value. Conversely, the latter, with reference to the truth, is an overestimate of 11 per cent.

The influence of oxalate cannot be dismissed without reference to its so called "bleaching" effect upon the creatinine color.

That excessive amounts of oxalate may be a source of danger in this respect was pointed out by Folin in his original description of the method; but according to Gettler⁷ the effect is noticeable even when the quantity employed is less than double the prescribed proportion. Although we have tested the influence of oxalate upon the color in three different ways, we are unable to confirm this statement.

Thus, in one experiment a saturated picric acid solution containing 0.5 mg. of creatinine per 100 cc. (a "2.5 mg." standard) was divided into two parts. To one was added 20 per cent potassium oxalate in the proportion of ten drops per 100 cc., which is rather more than the amount which a blood filtrate would regularly contain; to the other was added an equal proportion of water. Both were treated with the proper quantity of alkali, and immediately compared with one another in the colorimeter. Neither at the first nor within the course of the next 3 hours was the slightest difference between them to be detected. The oxalate had no effect whatever.

In another experiment we measured from a specimen of defibrinated ox blood four 10 cc. portions, to which we added respectively 0, 3, 6, and 12 drops of 20 per cent oxalate solution. A creatinine determination was then carried out on each. The results were practically identical, for they appeared to contain respectively 1.60, 1.65, 1.65, and 1.60 mg. per 100 cc. According to this experiment as much as four times the usual amount of oxalate may be employed with very little, if any, effect upon the result.

A third test consisted in directing human blood from an arm vein partly into a vessel containing a little 20 per cent oxalate solution, partly into one supplied with a flake or two of hirudin. Each part was divided into a smaller and a larger fraction, and from the latter the plasma was separated in the centrifuge. Each specimen of whole blood and of plasma thus obtained was finally analyzed for creatinine by the Myers' procedure. The whole experiment was repeated with a second subject. In each determination the standard was a "1 mg." one set at 20 mm. The colorimeter readings are shown in Table VI. It will be seen that the presence or absence of oxalate has little effect upon any one of the results. Such differences as appear are not greater than can be readily account-

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ed for by the merely diluent action of the oxalate solution added. We are therefore of the opinion that in all ordinary circumstances this effect of dilution is the only one that need be taken into account.

TABLE VI.
Effect of Oxalate upon the Creatinine Color in Blood and Plasma.

	1 mg. standard.	Hirudinised.	Oxalated.
	mm.	mm.	mm.
Whole blood A.....	20	14.0	14.7
Plasma A.....	20	18.1	18.5
Whole blood B.....	20	13.7	14.7
Plasma B.....	20	17.4	18.2

DISCUSSION AND CONCLUSIONS.

It has been shown that of the four or, including the Myers' modification, five applications of the Folin method for blood creatinine only one meets successfully each of the tests which we proposed. While it is admitted that these tests are incapable by themselves of furnishing any absolute demonstration of accuracy, one may nevertheless, in accordance with our main argument, infer that the colorimetric part at least of that particular application is in all probability free from serious error. The case thus specially distinguished from the others is that of the determination of *preformed creatinine in plasma*. Since for the same case it has been further shown, that the merely manipulative imperfections of the technique must all but neutralize each other's influence, the substantial accuracy of results upon the creatinine content of plasma would seem to be established. The net effect of all the relatively unimportant sources of error detected (glucose, protein precipitate, and dilution by oxalate) can hardly amount to 10 per cent, and may be very much less.

In all the other circumstances in which the colorimetric determination of creatinine is applied to blood analysis, it fails more or less completely to satisfy two out of the three requirements laid down. Failure in one would be sufficient to convict the method of inaccuracy. As to the relative extent to which its usefulness is affected in the different cases, both of the available criteria yield, it has been pointed out, identical implications.

In the case of the *preformed creatinine of whole blood* the error is considerable, yet perhaps not, in view of all the circumstances, immoderately large. The time curve of the reaction indicates, we have seen, that colorimetry exaggerates the creatinine content of the blood by, on the average, 36.5 per cent. Moreover, the minor sources of error inherent in the operations preliminary to the actual determination cannot with whole blood be assumed to cancel one another. Rather they involve a further error in the positive direction amounting to something like 11 per cent. The cumulative effect of these errors will be that the preformed creatinine of whole blood will be estimated at 150 per cent of its true value.¹⁸ Conversely, to reach the probable true value from the observed, one must diminish the latter on the average by one-third. It may be noted that if this conclusion be applied to the average creatinine content of the eleven bloods from which we constructed our time curve, it will be reduced from 1.57 to 1.05, which is identical with the average creatinine content of the plasmas.

The opinion that the Folin method exaggerates the creatinine content of whole blood has already been expressed, or at least implied, by Wilson and Plass.⁸ In support of it they offer very little positive evidence. As to the extent of the exaggeration they content themselves with the unsupported statement or assumption that the differences between the observed creatinine contents of whole blood and plasma are "sufficiently small to be ascribed to the anomalous color development when whole blood is used." Our observations place these, as we believe, perfectly correct conclusions upon a decidedly firmer basis, and furnish real evidence on which to discuss the question of the distribution of creatinine in the blood. To that question we propose to return in a later communication.

Our estimate of the probable error of whole blood creatinine determinations applies only to the original technique of Folin.

¹⁸ Of course it should not be forgotten that our manner of using the Folin method differs in one respect from the original; namely, in the use of interpretative curves. With most normal bloods this has the effect of giving a higher result than the usual calculation according to the rule of inverse proportionality. The error of the absolutely unmodified Folin procedure will therefore generally be less than the estimate applicable to our own results.

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When the blood is laked, all our observations agree in indicating that the error is considerably greater. Even the dilution test, successfully passed by all other cases, fails in this particular instance. The average error which results from laking cannot fairly be estimated from the limited number of data we possess; but the error of individual cases has been found to exceed 100 per cent.

For the accurate determination of *creatinine* in *whole blood* the method of Folin would appear, on the evidence we present, to be quite inadequate. The experiments bearing upon this point are so consistent and so definite in their significance, that they may be regarded, in spite of their limited number, as thoroughly convincing. Calculations based upon the time curve of the reaction in autoclaved blood suggest that the true creatine content of whole blood may be only one-half of that indicated by the method. The absolute magnitude of such an error would amount to several mg. per 100 cc.

By devising new methods presumably free from the objections to which Folin's is liable, Wilson and Plass,⁸ as well as Greenwald,^{9,10} have already reached upon the question of blood creatine the same conclusion. Our results are to be regarded therefore mainly as a confirmation, from a different angle, of theirs. The new methods agree with each other, and with our own deductions, in indicating for the old one an error of the order of 100 per cent.

Plasma, as regards the determination of its *creatinine*, would appear on the evidence of our tests to be on no better footing than whole blood. The probable error, as we have calculated it, is, absolutely considered, smaller than in the latter, but on account of the low creatine content of plasma relatively greater. It is indicated that the results of the Folin method in this application may be nearly four times too high. On this point our conclusion is in conflict with that of Wilson and Plass,⁸ who say in reference to it that "the agreement of the results" (according to Folin) "with those of the acetic acid procedure . . . lends support to the accuracy of the method." It may, however,

¹⁰ We are indebted to a private communication for the information that eight human bloods, of which the creatine content according to the method of Folin varied from 2.5 to 8.5 (average 5.3) mg. per 100 cc., contained according to that of Greenwald only 2.3 to 4.1 (average 3.3).

be pointed out that the agreement of which they speak does not appear in their published results to be a very close one. In one case (mixed specimen) the Folin method gives eight times as much as the 0.1 mg. given by the other. In two more (W, H) where the acetic acid procedure shows no creatine at all, the Folin indicates 0.4 to 0.6 mg. These are magnitudes which are absolutely small enough, but relatively to those with which Wilson and Plass are dealing by no means inconsiderable. In only four out of the nine human plasmas which they examined by both methods do the results actually agree.

It remains to be said, in conclusion, that while the verdict just passed upon certain applications of the Folin method for creatinine to blood analysis is, from the point of view of the strictest accuracy, far from favorable, it is by no means to be regarded as wholly condemnatory. Especially is this the case with the determination of preformed creatinine in whole blood. A 50 per cent error might here, according to the circumstances, have a very great significance or none at all. In a problem like that of creatinine distribution it becomes of crucial importance. For most of the purposes to which blood creatinine estimations have been applied it is of no consequence whatever. To the clinician it matters little whether a patient's blood contain 1.5 mg. of creatinine per 100 cc. or only 1.0; he is concerned to discover variations of much greater magnitude. The clinical usefulness of the method is the less likely to be affected, because the relative importance of the error probably, as indicated by our own observations on the patient K, diminishes as the creatinine concentration increases. Even with the technique of Myers, which proved to be quite useless for certain purposes we have had in view, it is possible, as its originator has abundantly proved, to obtain results of the greatest interest and importance. Only when it is a question of attaining the utmost possible accuracy will it become necessary or advisable to make the determination upon the plasma in preference to the blood.

In this aspect of the matter even the method for creatine, which in comparison with later improvements has the merit at least of simplicity, may possess a certain restricted usefulness. It is probable enough that its results vary in a general way with the true creatine content of the blood. The relative constancy

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of the results obtained with normal subjects, and the generally observed increase in pathological conditions associated with retention of other blood constituents, would lend support to such an assumption. A series of comparative analyses in which the Folin method for creatine has been employed may therefore easily reveal real fluctuations in the concentration of that substance. Only, in order that this should be possible, or that different series should be in any sense comparable, it will be essential, as our experiments demonstrate, that the same standard be employed in every single determination.

SUMMARY.

Time curves are presented showing the rate at which the color utilized as the basis of a creatinine determination develops, not only in pure aqueous solutions, but also in the various circumstances in which it is applied to the analysis of blood. By comparison of these curves it is shown to be probable that:

1. The only substance in plasma capable of simulating the reaction for creatinine is glucose, and its influence upon the determination is too small to have much practical importance.

2. Whole blood contains in addition an unknown substance which, although reacting more slowly than creatinine, contributes in the Folin technique an appreciable fraction of the total color developing within 10 minutes.

3. The amount of this substance passing into the protein-free filtrate is greatly increased on laking the blood.

4. Autoclaved blood or plasma filtrates contain a substance reacting instantaneously upon the addition of alkali in such a manner as to simulate relatively substantial quantities of creatinine.

These deductions are corroborated by the way in which a change of standard affects the outcome of the several determinations studied.

After weighing the probable effect of certain manipulative imperfections of the method, it is finally concluded that:

1. The Folin method determines the preformed creatinine of plasma with a satisfactory approximation to accuracy.

2. The results of the same method upon the preformed creatinine of whole blood are on the average about 50 per cent higher than the truth.¹⁸

- . The technique of Myers leads to a still greater exaggeration of the preformed creatinine of blood.
- . The Folin method for creatine, whether applied to whole blood or to plasma, gives decidedly erroneous results, liable to be in the former case about twice, and in the latter about four times, as high as the amount actually present.

A MICRO METHOD FOR THE DETERMINATION OF CALCIUM AND MAGNESIUM IN BLOOD SERUM.

By W. McKIM MARRIOTT AND JOHN HOWLAND.

(From the Department of Pediatrics, Johns Hopkins University, Baltimore.)

(Received for publication, October 1, 1917.)

The methods described below¹ were devised especially for the study of the composition of the blood of infants suffering from rickets and tetany, and are not recommended as substitutes for the usual gravimetric procedures where a sufficient amount of material is available. By these methods it is possible to determine calcium and magnesium in 2 cc. samples of serum with a maximum error of less than 5 per cent, a degree of accuracy entirely sufficient for the purposes for which the methods were devised.

Principle of the Methods.

The methods depend upon the fact that solutions of ferric thiocyanate are decolorized by oxalates and by phosphates. Calcium is precipitated as the oxalate and magnesium as the ammonium magnesium phosphate; the precipitates are dissolved in acid and added to solutions of ferric thiocyanate, the degree of decolorization resulting being determined by comparison in small Nessler tubes.

Technique of the Calcium Method.

2 cc. of clear serum are measured into a 50 cc. conical beaker, 10 cc. of concentrated nitric acid are added, and the beaker is heated on an electric stove just below the boiling point for 2 or 3 hours. The heat is then increased and the acid evaporated down to about 0.5 cc. Sputtering must be guarded against and the contents must not go completely to dryness.² The sides of

¹ A preliminary description of these methods appeared in the Proceedings of the American Society of Biological Chemists, *J. Biol. Chem.*, 1916, xxiv, p. xviii.

² If charring occurs, more acid must be added and evaporation repeated.

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the beaker are washed down with 2 or 3 cc. of water and a drop of phenolsulfonephthalein is added as an indicator. Dilute ammonia is added drop by drop until alkaline.³ The beaker is then replaced on the stove and heated until the excess of ammonia is removed, as shown by a change in color of the indicator and the fact that only a faint odor of ammonia remains. Unless a very great excess of ammonia has been added, 2 or 3 minutes at medium heat is usually sufficient. While still hot 1 cc. of a solution of 1.25 per cent oxalic acid in 0.25 N hydrochloric acid is added drop by drop and with stirring. The beaker is removed from the stove and when cool 0.5 cc. of sodium acetate solution (20 per cent)⁴ is added slowly and with stirring. The solution is allowed to stand over night. This method of precipitation, which is essentially that advised by McCrudden,⁵ results in a granular precipitate of calcium oxalate, that can be readily filtered without loss.

Filtration is carried out on a 10 cc. Gooch crucible, the mat being especially prepared as follows: A small disc of filter paper is first placed in the bottom of the crucible, asbestos soup is poured on to make a fairly thick mat, another disc of filter paper is laid on and then a little more asbestos, and finally a suspension of purified barium sulfate. This latter serves to make evident any leaks in the crucible and also to close the pores.

The calcium oxalate precipitate is washed into the crucible and beaker and crucible are washed eight times, each time with approximately 5 cc. of 1 per cent ammonia (one part concentrated ammonia in 100 of water), then once with 95 per cent alcohol, containing just enough ammonia to be alkaline, and finally once with ether.⁶ The suction is best diminished when the ether is poured on the crucible so as to prevent excessively rapid filtration. After all the ether has passed through the filter, the suction is increased and after 5 or 10 minutes the mat is quite dry.

³ The indicator changes from an eosin pink to yellow and then to reddish purple, the latter color being the alkaline end-point.

⁴ This refers to the anhydrous salt. If crystalline sodium acetate is used the solution should be made up to a strength of 35 per cent.

⁵ McCrudden, F. H., *J. Biol. Chem.*, 1909-10, vii, 83, 201; 1911-12, x, 187.

⁶ Ether which contains acid should be redistilled over sodium hydroxide before using.

The crucible is returned to the beaker and 10 cc. of 0.2 N hydrochloric acid are run into the crucible and allowed to percolate through. The beaker is covered by a piece of rubber dam held in place by a rubber band or put in a desiccator over water, in order to prevent evaporation. After standing several hours or over night the asbestos is thoroughly stirred up in the acid and the whole suspension transferred to a tube and centrifuged. An aliquot portion of the clear supernatant fluid (usually 6 cc.) is pipetted off and used for the colorimetric determination.

Colorimetric Comparison.

Small Nessler tubes approximately 120 mm. long and 10 mm. internal diameter are used. They are graduated at 10 cc., and the bores of the tubes should be such that the graduation on each tube should be within 2 mm. of the graduation on any other tube in the set. Round-bottomed tubes may be used, but those with flat bottoms are preferable. Aliquot portions of the oxalate solution are measured into the tubes. Portions of a standard calcium oxalate solution⁷ are measured into other tubes. Then to each tube are added 2 cc. of ferric thiocyanate⁸ solution accurately measured with an Ostwald pipette. Each tube is filled to the mark with 0.2 N hydrochloric acid, and the contents are mixed by inverting several times, the ends of the tubes being closed with a clean rubber stopper.

Color comparisons are made by looking lengthwise through the tubes against a dull white background. Readings are first made

⁷ The standard calcium oxalate solution is made by dissolving 0.0630 gm. of pure oxalic acid ($\text{H}_2\text{C}_2\text{O}_4 \cdot 2\text{H}_2\text{O}$) in water. To this are added 200 cc. of N hydrochloric acid and sufficient calcium chloride solution to correspond to 20 mg. of calcium. The whole is made up to 1 liter with water. 1 cc. of this solution is equivalent to 0.02 mg. of calcium as calcium oxalate.

⁸ The ferric thiocyanate solution is made from two solutions which are mixed $\frac{1}{2}$ hour before use. Solution A is 0.3 per cent ammonium thiocyanate. Solution B is 0.3 per cent ferric chloride, made up from the salt with its contained water of crystallization, adding a few drops of acid, if necessary, to clear the solution. 5 cc. portions of Solutions A and B are mixed and the whole is diluted to 25 cc. with water. This is the proper dilution for use with ordinary serum, but when smaller amounts of calcium are present the solution should be more dilute; with larger amounts it should be more concentrated.

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against standard oxalate solutions differing from each other by 0.5 cc., interpolating when necessary. Extra standards may then be made up to obtain more exact results.

The calculation is simple, as, for example, 2 cc. of serum were used, and a 6 cc. aliquot portion of the oxalate solution was taken. The color in the comparison tube, after adding thiocyanate and diluting to the mark, was found to match that corresponding to 6.3 cc. of the standard oxalate solution; then

$$6.3 \times .02 \times \frac{10}{6} \times \frac{100}{2} = 10.5 \text{ mg. per 100 cc. of serum.}$$

It is necessary to run blank determinations on the reagents used and to make allowance for any blank found to be present. In our experience we have never encountered a blank amounting to more than 0.1 cc. of the standard oxalate solution. Other workers, however, have informed us that some samples of nitric acid contain appreciable amounts of calcium. We have used Baker's analyzed nitric acid and have found it free from significant amounts of calcium.

Results of Calcium Determinations.

Solutions of calcium chloride containing known amounts of calcium, as determined gravimetrically, were suitably diluted, and portions containing amounts of calcium comparable to the amounts present in the serum used were analyzed according to the method described above. The range of accuracy on solutions containing from 0.1 to 0.2 mg. of calcium was usually within 2 per cent of the theoretical values. Exceptionally a variation of as much as 5 per cent from the theoretical value was encountered; the average of duplicate determinations, however, almost invariably agreed within 2 per cent of the theoretical.

Known amounts of calcium (0.1 to 0.2 mg.) added to 2 cc. samples of serum were quantitatively recovered with the same degree of accuracy as in pure solution.

The serum of normal adults, of infants, and of dogs was found to contain from 9.5 to 11.5 mg. of calcium per 100 cc. In certain pathological conditions, such as infantile tetany, and terminal uremic conditions, great reductions in the calcium content of the serum were observed.

Serum has been used instead of whole blood since it is known that the corpuscles contain no calcium, and that the fibrin clot contains only a very small and fairly constant amount of calcium. If whole blood were used the calcium content would vary inversely with the corpuscular count, other things being equal.

Technique of the Magnesium Method.

When it is desired to determine magnesium in the same sample of serum used for calcium determination, the procedure for calcium has to be modified somewhat. After precipitation of the calcium oxalate, as previously described, the contents of the beaker are rinsed into a conical centrifuge tube and centrifuged for about 10 minutes. The clear liquid is syphoned off⁹ into a small casserole or a platinum dish and used for magnesium determination. The calcium oxalate remaining in the centrifuge tube is dissolved in 0.5 cc. of concentrated nitric acid. This is best accomplished by first adding the acid and then 5 or 10 cc. of water; the tube is then heated by immersion in a beaker of hot water and the contents are stirred by bubbling air through the tube by means of a capillary glass tube reaching to the bottom of the centrifuge tube and connected with a rubber bulb. The contents of the tube are then transferred to the beaker and evaporated to a volume of about 5 cc.; the calcium is then reprecipitated and determined exactly as described above.

The liquid syphoned off into the dish contains the magnesium¹⁰ present, to this is added 0.5 cc. of concentrated sulfuric acid, and the liquid is evaporated to dryness on an electric stove. Ashing is completed over a Méker burner. If a small amount of black residue remains, a drop of sulfuric acid is added, after cooling, and the ashing repeated. To the residue is added 0.5 cc. of con-

⁹ Syphoning is accomplished by means of a small glass tube, one end of which is drawn to a short capillary point and bent upwards. This form of tip prevents the sucking up of any precipitate and permits the removal of almost all of the liquid. The syphon tube is passed through a small double-bored rubber stopper, which fits the centrifuge tube. A short glass tube passes through the other hole in the stopper. The syphon is started by blowing into this tube or by the use of an atomizer bulb.

¹⁰ Approximately 0.2 cc. of liquid remain in the centrifuge tube, and this amount, which is about 2 per cent of the total, may be allowed for in the final calculation, if extreme accuracy is desired.

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centrated hydrochloric acid and a little water. The dish is warmed and the contents are transferred to a small (25 cc.) beaker with several washings of hot water. The beaker is put on an electric stove and the contents are evaporated to a volume of about 3 cc., a drop of phenolsulfonephthalein is added, and then 1 cc. of ammonium phosphate solution.¹¹ 1 cc. of concentrated ammonia is run in with stirring and the beaker allowed to stand over night. Crystals of ammonium magnesium phosphate separate out on the sides and bottom of the beaker.

The contents of the beaker are transferred to a conical 15 cc. centrifuge tube and centrifuged for a couple of minutes. The liquid is syphoned off in the manner above described and the precipitate in beaker and tube washed with 10 per cent ammonia (one part concentrated ammonia to nine parts of water), the precipitate in the tube being stirred up each time with the capillary blowing tube. It is unnecessary to dislodge the precipitate from the sides of the beaker. The washing is repeated four times, each time with from 6 to 10 cc. of ammonia. The precipitate is finally washed once with 95 per cent alcohol made just alkaline with ammonia. This is syphoned off and tube and beaker are dried in an air oven at about 60°C.

After drying, the contents of beaker and tube are dissolved in 10 cc. of 0.01 N hydrochloric acid and an aliquot portion is pipetted off for colorimetric determination.

The colorimetric comparison is done in the same way as in the calcium determination with the exception that the thiocyanate solution used is diluted to 40 or 50 cc. instead of to 25, a standard solution of magnesium ammonium phosphate¹² is used instead of

¹¹ Ammonium phosphate solution is made as follows: 25 gm. $(\text{NH}_4)_2\text{PO}_4$ are dissolved in 250 cc. H_2O . 25 cc. of concentrated ammonia are added and the mixture is allowed to stand over night. The following day it is filtered, the filtrate is boiled to remove the excess of ammonia, cooled, and made up to 250 cc.

¹² This solution is made by dissolving 0.102 gm. of air-dried magnesium ammonium phosphate ($\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$) in 100 cc. of 0.1 N hydrochloric acid and diluting to 1 liter with water. Of this solution 1 cc. is equivalent to 0.01 mg. of magnesium. Magnesium ammonium phosphate loses water of crystallization when heated and must therefore be dried at room temperature. Commercial preparations of the salt are generally unreliable; it should be prepared by precipitation of pure solutions. See Jones, W., *J. Biol. Chem.*, 1916, xxv, 87.

one of calcium oxalate, and dilutions in the Nessler tubes are made with 0.01 N hydrochloric acid. The calculation of results is as follows: If 2 cc. of serum were used and a 6 cc. aliquot portion of the solution of the precipitate were taken, and the color found to be the same as that of 3.2 cc. of the standard magnesium solution, then

$$3.2 \times 0.01 \times \frac{10}{6} \times \frac{100}{2} = 2.66 \text{ mg. magnesium per 100 cc. of serum.}$$

Results of Magnesium Determinations.

Solutions containing known amounts of calcium and magnesium were analyzed as described above, and amounts of magnesium ranging from 0.02 to 0.04 mg. were recovered practically quantitatively; the average of duplicate determinations being within 5 per cent of the theoretical value. Similar amounts of magnesium added to serum were quantitatively recovered, the presence of organic matter having no effect on the accuracy of the determination. Human blood serum was found to contain from 2.2 to 3.5 mg. of magnesium per 100 cc.

On account of the extremely small amounts of magnesium present in the serum and the number of manipulations necessary, we feel that the method is hardly as accurate as the calcium method but that it is possible to obtain sufficiently accurate results for comparative purposes.

Detailed results of calcium and magnesium determinations in the serum of infants suffering from rickets and tetany will appear shortly in *The Quarterly Journal of Medicine*.

A MICRO METHOD FOR THE DETERMINATION OF INORGANIC PHOSPHATES IN THE BLOOD SERUM.

By W. McKIM MARRIOTT AND F. H. HAESSLER.

(From the Department of Pediatrics, Johns Hopkins University, Baltimore.)

(Received for publication, October 1, 1917.)

The method here described was devised in order to determine whether or not a retention of inorganic phosphates, with a resulting increase of these substances in the blood serum, occurs in patients suffering from certain types of nephritis. Results obtained by the use of the method in such cases have already appeared.¹

The method depends upon the same principle as that for magnesium determination described in the preceding paper;² namely, the decolorization of solutions of ferric thiocyanate by an acid solution of ammonium magnesium phosphate. The phosphates of the serum are precipitated directly by means of magnesia mixture.

Technique of the Method.

Dilute 1 cc. of clear serum with about 5 cc. of water in a small beaker. Add 2 drops of 0.1 N hydrochloric acid and 1 cc. of magnesia mixture.³ Then with stirring, run in slowly 2 cc. of 10 per cent ammonia (one part concentrated ammonia to nine parts of water), and allow to stand over night in a cool place.

The precipitate is filtered off on a small Gooch crucible, the mat being prepared as for the calcium determination.² Wash the

¹ A preliminary report of the method appeared in the Proceedings of the American Society of Biological Chemists, *J. Biol. Chem.*, 1916, xxiv, p. xviii. For results in nephritis cases see Marriott, W. McK., and Howland, J., *Arch. Int. Med.*, 1916, xviii, 708.

² Marriott and Howland, *J. Biol. Chem.*, 1917, xxxii, 233.

³ Dissolve 10 gm. of magnesium chloride (sticks) and 5 gm. of ammonium chloride in 250 cc. of water; add 10 cc. of concentrated ammonia. Allow to stand over night, filter, neutralize with hydrochloric acid, using phenol-sulfonephthalein as an indicator, and make up to 500 cc. with water.

precipitate and beaker four times, each time with about 5 cc. of the 10 per cent ammonia, then once with 95 per cent alcohol made just alkaline with ammonia, and finally with 5 cc. of ether. Suction is kept on for several minutes in order to dry the mat. The crucible is then returned to the beaker and 10 cc. of 0.01 N hydrochloric acid are run in and allowed to percolate through the crucible. The beaker is covered with a rubber dam or put in a desiccator over water and allowed to stand for a couple of hours or over night. The asbestos is then stirred up in the acid and the suspension transferred to a centrifuge tube and centrifuged. A 6 cc. aliquot portion of the clear liquid is pipetted off and used for colorimetric determination. This is carried out exactly as the magnesium determination, except that a solution of magnesium ammonium phosphate of a somewhat different strength is used as a standard.⁴

The calculation of results is as follows: If 1 cc. of serum were used and a 6 cc. aliquot portion of the dissolved precipitate taken for colorimetric determination, the color in the Nessler tube corresponding to that resulting from the addition of 0.9 cc. of the standard phosphate solution, then

$$0.9 \times 0.02 \times \frac{10}{6} \times \frac{100}{1} = \frac{3.0 \text{ mg. phosphorus}}{\text{per 100 gm. of serum.}}$$

RESULTS.

Solutions of inorganic phosphates containing from 0.01 to 0.1 mg. of phosphorus were analyzed according to the method described and quantitative results obtained. Similar amounts of phosphate added to the serum were quantitatively recovered, the presence of organic matter apparently having no effect in preventing the precipitation of the phosphate. We further found that lecithin in amounts comparable to that occurring in serum, yields no inorganic phosphate when treated according to the method described.

⁴ This solution is made by dissolving 0.1584 gm. of air-dried magnesium ammonium phosphate ($\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$) in 100 cc. of 0.1 N hydrochloric acid and diluting to 1 liter with water. 1 cc. of this solution is equivalent to 0.02 mg. of phosphorus.

The method is not applicable to whole blood, without modification, as the corpuscular debris renders filtration extremely slow. In some instances, the serum, if allowed to stand for too long a time before filtration, filters very slowly. In such instances the liquid may be centrifuged, the precipitate dissolved in dilute acid, and the phosphates reprecipitated.

Applying the method to the determination of inorganic phosphates in the serum of normal individuals we have found from 1.0 to 3.5 mg. per 100 cc. of serum. In the serum of nephritics suffering from acidosis amounts of inorganic phosphorus as high as 20 mg. per 100 cc. of serum have been determined.

IRON AS AN ANTIDOTE TO COTTONSEED MEAL INJURY.*

BY W. A. WITHERS AND FRANK E. CARRUTH.

(*From the Chemical Division of the North Carolina Agricultural Experiment
Station, West Raleigh.*)

(Received for publication, September 5, 1917.)

As a result of feeding rabbits¹ successfully 106 days on cottonseed meal with ferric ammonium citrate, several experiments were conducted with pigs, using iron salts to prevent cottonseed meal poisoning. It was desired to learn whether cottonseed meal could be used safely in practice as a pig feed. These experiments are incomplete from a practical standpoint. They are of interest, however, as they relate to the question whether cottonseed meal injury is due to the presence of a harmful substance, or whether, as is claimed by some, it is due to inadequate diets or erroneous methods of feeding. It is believed that by using iron salts with cottonseed meal the toxic factor is more or less counteracted, thus allowing the diet to approach its true feeding value.

Although the addition of iron salts may not be recommended as a successful way for the practical feeder to overcome cottonseed meal poisoning, the experiments show that with iron salts much

* The feeding experiments with swine were conducted by the Animal Husbandry Division under the supervision of R. S. Curtis and later, D. T. Gray, Chief of the Division. The postmortem examinations were made by Dr. G. A. Roberts, Veterinarian of the Experiment Station. His observations are recorded in the *35th Ann. Rep., North Carolina Agric. Exp. Station*, 1911-12, 27.

¹ Rabbits are usually very quickly affected by cottonseed meal, even though long cooked. They are made sick in 1 to 4 weeks, depending on the toxicity of the meal. The experiments with rabbits were described by Withers and Brewster (*J. Biol. Chem.*, 1913, xv, 161). Regarding the rabbits receiving the iron salts, Withers and Brewster state: "Each of these rabbits was alive and normal after 58 days' feeding, each having consumed 870 gm. of cottonseed meal and 19.95 gm. of iron salts."

larger quantities of cottonseed meal have been fed and better gains made² than would have been possible otherwise, and that in many cases death has been averted.

Further evidence of the presence of a toxic substance in the meal is the fact that extraction with alcoholic alkali removes the toxicity (rabbit experiments³).

We have assumed that this is due to the formation of an insoluble iron salt of gossypol or one of its derivatives together with the catalytic acceleration of the oxidation of gossypol and perhaps a tonic action of iron on the system. Possibly with a liberal supply of iron, the blood of the animal is better enabled to convert gossypol, in whatever form absorbed, into non-toxic products. This action of iron salts has no adequate explanation from the deficiency standpoint. Treatment of cottonseed meal with iron salts causes the meal to turn brownish black, indicating a reaction with some component of the meal. A similar color may be produced by the action of iron salts on a solution of gossypol.

EXPERIMENTAL.

Three lots of six pigs each were fed cottonseed meal with corn meal, 1:3, and two other lots of three each were fed equal parts of cottonseed meal and corn meal. It was planned to feed cottonseed meal to the extent of 1 per cent body weight daily in the first three lots, and 2 per cent daily in the other lots, with a correspondingly large amount of iron salt. As the experiment progressed the amounts fed fell below the 1 per cent and 2 per cent basis.

The pigs were kept in dry lots about 50 by 100 feet. The conditions were much more favorable than in the experiments to be described later, in which pigs were confined in pens under a barn during the winter.

The percentage composition of the diets was as follows:

Lot No.....	243	239	242	258	259
Cottonseed meal.....	25	25	25	50	50
Corn meal.....	75	75	75	50	50

² This gain, of course, is due to the larger intake of food. Up to the time pigs are affected by cottonseed meal the gains are often not greatly different from those receiving iron salts.

³ Withers, W. A., and Ray, B. J., *Science*, 1912, xxxvi, 31.

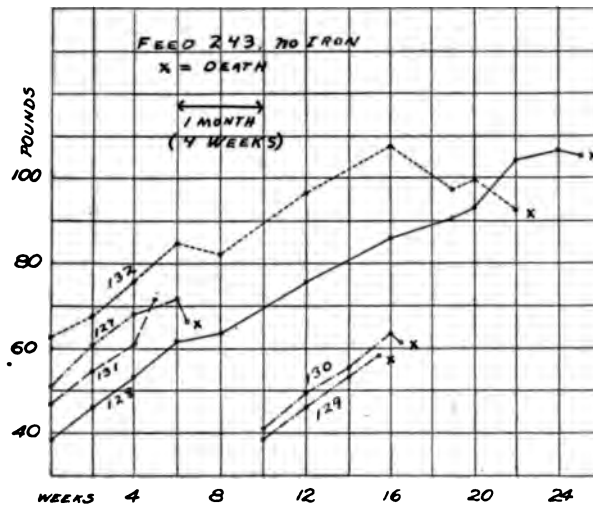


CHART I. Control lot. Six pigs on a diet of cottonseed meal and corn meal without iron all died before the experiment ended. Four deaths were typical sudden deaths.

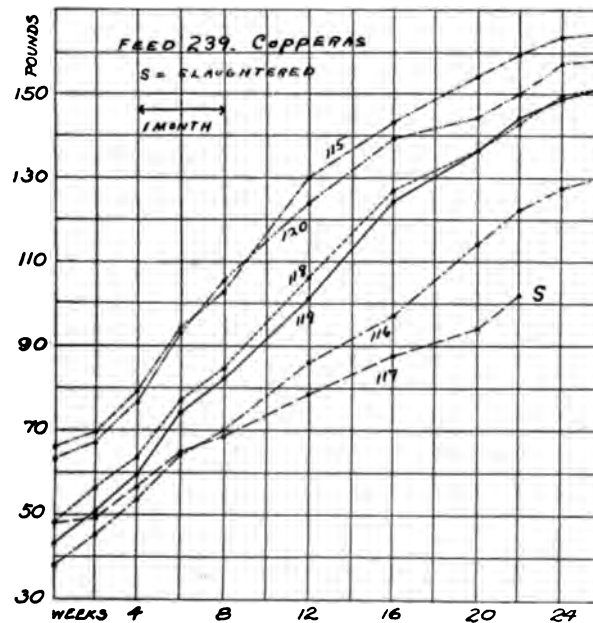


CHART II. Cottonseed meal with corn meal (1:3) with copperas solution. Five of the six pigs finished the 180 day experiment. The other was slaughtered after 154 days. No symptoms of cottonseed meal poisoning were evident on postmortem examination.

Lot 243 received to each 4 pounds of feed 1 gallon of water.

Lot 239 received to each 4 pounds of feed 1 gallon of a copperas solution (1 pound $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ dissolved in 50 gallons of water). 1 gallon contained approximately 9 gm. of copperas, or 2.4 gm. of iron.

Lot 242 received a similar solution containing approximately the same weight of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$.

Lot 258, three pregnant sows, received 1 gallon of a copperas solution four times as strong as that used in Lot 239. This furnished twice as much iron to each pound of cottonseed meal.

Lot 259, three pregnant sows, received a solution of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ of similar strength.

These sows except Nos. 133 and 136 farrowed between the 10th and 12th weeks. Up to this time they made excellent gains and after farrowing steadily fell off. Below are given the daily gain up to the 63rd day and the other data regarding the young.

Sow No.....	134	135	136	137	138
Daily gain (63 days)...	0.74	0.79	0.87	0.71	0.82
Number of young born.	2	5	0	3	4
Number of young living					
3 weeks or more.....	2	4	0	2	4

These results with iron salts in connection with cottonseed meal have an interesting application in regard to the theory of nutritive deficiencies of cottonseed meal as fed in these diets. The toxic factor, being more or less completely inhibited by the iron salt, leaves any deficiency of the diet to show its effect on the growth. In other words, the results of the copperas experiment show that by eliminating the toxic factor we have no evidence of nutritive deficiency in cottonseed meal great enough to produce failure in a relatively short period.

The toxic factor is probably not so well overcome here as in subsequent experiments in which cottonseed kernels were extracted by ether and fed.

The growth curves for these experiments are shown in Charts I, II, III, and IV.

Experiment 2.—All the pigs which survived the previous 180 day experiment, together with some shoats of about 100 pounds' weight, were started immediately at the close of the first experiment on a second experiment to test the value of iron salts. This experiment continued 121 days. It will be seen that in some cases pigs were fed on cottonseed meal for a total of 301 days.

TABLE I.
Experiment 1.

Pig No.	Weight.				Result.	Duration.
	Initial.	Final.	Daily gain.	Cotton-seed meal eaten.		
Feed 243. Check lot. No iron.						
	<i>lbs.</i>	<i>lbs.</i>	<i>lbs.</i>	<i>lbs.</i>		<i>days</i>
127	51	66	0.33	25	Died.	46
128	38.5	105	0.37	79	"	177
129	38.5	58	0.50	19.5	"	38
130	41	61.5	0.44	22	"	45
131	47	72	0.71	18	"	35
132	62.5	92.5	0.19	109.5	"	154
Average.	46.4	75.8	0.35	45.5		83
Feed 239. Copperas lot.						
115	66	164	0.54	128	Discontinued.	180
116	38	130	0.51	128	"	180
117	48	101.5	0.35	71	Slaughtered.	154
118	48	151	0.57	128	Discontinued.	180
119	43	151	0.60	128	"	180
120	63	158	0.53	128	"	180
Average.	51	142.6	0.52	118		176
Feed 242. Ferric chloride lot.						
121	63	163	0.56	128	Discontinued.	180
122	41	126.5	0.46	128	"	180
123	33	63.5	0.49	31.5	Died.	62
124	35	73	0.25	23	Slaughtered.	154
125	43.5	123.5	0.48	128	Died.	167
126	53	162	0.61	128	Discontinued.	180
Average.	44.7	118.6	0.48	94.4		154
Two other lots of sows were fed 152 days on equal parts of cottonseed meal and corn meal						
Feed 258. Stronger copperas solution.						
133	85	110	0.51	50.5	Died.	44
134	98	113.5	0.10	228	Discontinued.	152
135	98	117	0.13	228	"	152
Average.	93.7	113.5	0.17	169		116
Feed 259. Stronger ferric chloride solution.						
136	82.5	171	0.51	228	Discontinued.	152
137	92.5	120	0.18	228	"	152
138	84	139	0.36	228	"	152
Average.	86.3	143.3	0.37	228		

The ration was made up as before of 25 per cent cottonseed meal and 75 per cent corn meal. The various lots received in addition:

Feed 270. Control.

" 271. Hardwood ashes (pigs had access to boxes containing ashes) (copperas first 2 weeks).

" 272. Copperas solution and ashes as above.

" 273. Copperas solution.

The pigs were fed from troughs in large outdoor lots. The animals rooted considerably. The control lot consisted of fresh shoats which were fed until sickness occurred in all and then the survivors were transferred to the other lots. The data of the experiments are summarized in Tables II and III.

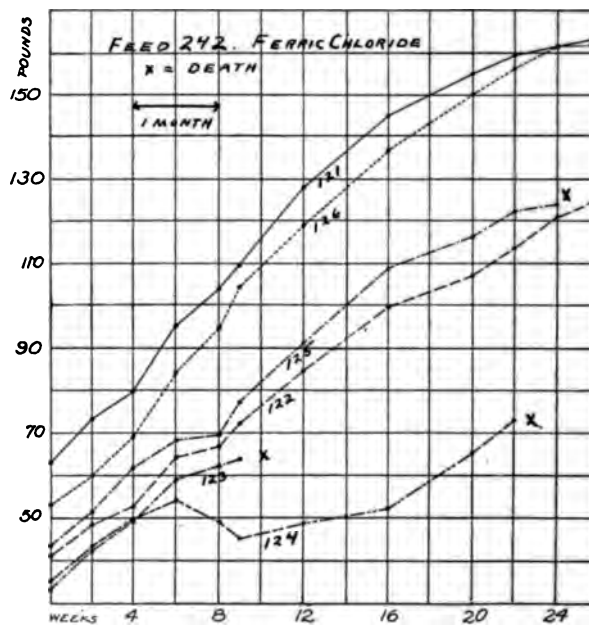


CHART III. Cottonseed meal and corn meal (1:3) with ferric chloride do not here seem as efficient as with copperas, but there is marked improvement over the control lot.

In some of these lots, especially in Lot 273, no conclusion regarding iron or ashes as antidotes may be drawn. The deaths in Lot 273 were probably due to results of the previous feeding. In all lots except No. 270 the meal was fed considerably below the basis of 1 per cent body weight. These animals were also much older than those of other experiments.

TABLE II.

Pig No.	Weight.				Result.	Duration.
	Initial.	Final.	Daily gain.	Cotton-seed meal eaten.*		
Feed 270. No antidote.						
	<i>lbs.</i>	<i>lbs.</i>	<i>lbs.</i>	<i>lbs.</i>		<i>days</i>
139	98	115	0.38	45	Died.	45
140	104	130	0.53	49	Transferred.	49
142	106	145	0.80	49	"	49
144	102	140	0.78	49	"	49
Average.	102.5	132.5	0.62	48		48
Feed 271. Ashes.						
120	158	225	0.55	121	Slaughtered.	121
121	163	260	0.80	121	Kept for breeding.	121
135	117	190	0.69	121	Slaughtered.	121
138	139	230	0.75	121	"	121
141	105	185	0.66	121	"	121
140	130	150	0.28	72	"	72
Average except 140.	136.4	218	0.67	121		
Feed 272. Copperas and ashes.						
115	164	275	0.92	121	Slaughtered.	121
118	151	205	0.45	121	"	121
122	126.5	190	0.52	121	"	121
136	171	245	0.61	121	"	121
143	100	165	0.54	121	"	121
142	145	200	0.46	72	"	72
Average except 142.	142.5	216	0.61			
Feed 273. Copperas.						
116	130	115	Lost.	119.5	Slaughtered.	121
119	151	152	0.13	7	Died.	8
126	162	285	1.02	119.5	Slaughtered.	121
134	113.5	195	0.67	119.5	"	121
137	125	140	0.26	54.5	Died.	57
144	140	142	0.29	7	"	7

* Estimated from weight of pigs and amounts fed to each lot.

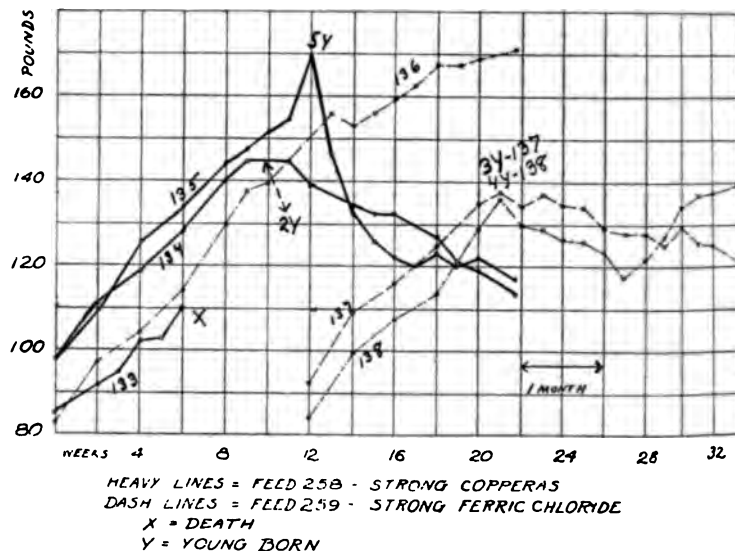


CHART IV. Pregnant sows were fed on a diet containing equal parts of corn meal and cottonseed meal. The iron solutions used on Feeds 258 and 259 furnished twice as much iron per pound of cottonseed meal as was used in Feeds 239 and 242. Disregarding the one early death these sows made excellent gains until farrowing time,—gains which one would not expect on a diet markedly lacking in one or more dietary factors.

It is of interest to compare the gains of the pigs which were also on the previous experiment and were in some cases, therefore, fed cottonseed meal 301 days consecutively.

Experiment 3.—In another experiment with four lots of nine pigs each, the pigs were confined in pens under a barn. Iron salts did not appear to be so advantageous, yet the deaths were considerably fewer among those pigs which received iron salts.

The experiment continued 94 days, after which the surviving pigs were slaughtered. The rate of feeding was practically the same—4 per cent of weight daily at the start—but this was not maintained. The grain ration in all cases was the same (cottonseed meal 25 per cent and corn meal 75 per cent) with the variations here noted. The lots received to each 4 pounds of feed:

- | | | |
|-----------|---|---|
| Feed 332. | 1 | gallon of water. |
| " 333. | 1 | " " copperas solution. |
| " 334. | 1 | " " ferric chloride solution. |
| " 335. | 1 | " " water with hardwood ashes supplied <i>ad libitum</i> as before. |

TABLE III.

Pig No.	Experiment 1.	Experiment 2.	Weight.			Total days fed.
			Initial.	Final.	Daily gain.	
			lbs.	lbs.	lbs.	
120	Copperas.	Ashes.	63	225	0.54	301
121	FeCl ₃ .	"	63	260	0.65	301
135	Stronger copperas.	"	98	190	0.30	301
138	Stronger FeCl ₃ .	"	84	230	0.48	301
115	Copperas.	Copperas and ashes.	66	275	0.69	301
118	"	"	48	205	0.52	301
122	FeCl ₃ .	"	41	190	0.49	301
136	Stronger FeCl ₃ .	"	82.5	245	0.54	301
116	Copperas.	Copperas.	38	115	0.27	301
119	"	"	43	152	0.58	188*
126	FeCl ₃ .	"	53	285	0.77	301
134	Stronger copperas.	"	98	195	0.32	301
137	Stronger FeCl ₃ .	"	92.5	140	0.20	237*

* Died.

Feed 332, control, was fatal to six pigs out of nine. Deaths occurred on the 36th, 40th, 43rd, 44th, 53rd, and 59th days.

Feed 333, copperas, was fatal to three pigs out of nine. Deaths occurred on the 40th, 41st, and 58th days.

Feed 334, ferric chloride, was fatal in only one case,—on the 59th day.

Feed 335, hardwood ashes, was fatal to seven pigs out of nine. Deaths occurred on the following days: 46th (2), 47th (2), 48th (2), and 59th.

Thus of a total of eighteen pigs receiving iron, only four died, while of a total of eighteen pigs without iron, thirteen deaths occurred.

These four deaths of pigs receiving iron showed that the mere addition of iron was not sufficient to overcome the toxic effects and deficiencies of this diet under such severe conditions.

Experiment 3 indicates clearly that the iron has some antidotal action.

Cottonseed Meal Injury

TABLE IV.
Experiment 3.

Pig No.	Weight.					Remarks.
	Initial.	Maxi- mum.*	Final.	Daily gain or loss.	Cotton- seed meal eaten.†	
Feed 332. No iron salt added.						
	<i>lbs.</i>	<i>lbs.</i>	<i>lbs.</i>	<i>lbs.</i>	<i>lbs.</i>	
49	148.5	171	149	0.01	84	Lived.
50	123	138	104	-0.36	53	Died, 53 days.
65	115	136	125	0.11	66	Lived.
66	124	143	139	0.17	71	"
67	78	85	78	0.00	27	Died, 43 days.
68	96.5	108	107	0.18	44	" 59 "
69	75	85	85	0.23	27	" 44 "
70	81	82	76	-0.14	25	" 36 "
71	89.5	98	98	0.25	31	" 40 "
Average.	103.4	116	107	0.05	47	
Feed 333. Copperas.						
72	95.5	152.5	133.5	0.40	82	Lived.
73	125.5		223.5	1.05	105	"
74	100	166.5	165	0.69	88	"
75	101.5	146.5	134.5	0.35	83	"
76	123		157	0.59	65	Died, 58 days.
77	68		104	0.38	56	Lived.
78	70.5		80	0.24	27	Died, 40 days.
79	67		85	0.44	26	" 41 "
80	78		129	0.54	65	Lived.
Average.	92.1		134.6	0.54	66	
Feed 334. Ferric chloride.						
81	136		241	1.12	112	Lived.
82	162		259	1.03	130	"
83	120		188	0.72	97	"
84	104		145	0.70	52	Died, 59 days.
85	85.5		129	0.46	68	Lived.
86	65		115	0.52	56	"
87	95		156	0.65	77	"
88	90		127	0.39	72	"
89	62		109	0.50	52	"
Average.	102.2		163.2	0.67	80	

TABLE IV—*Concluded.*

Weight.					Remarks.
Initial.	Maximum.*	Final.	Daily gain or loss.	Cottonseed meal eaten.†	
Feed 335. Hardwood ashes <i>ad libitum</i> .					
<i>lbs.</i>	<i>lbs.</i>	<i>lbs.</i>	<i>lbs.</i>	<i>lbs.</i>	
144		190	1.00	59	Died, 46 days.
138.5		202.5	0.68	94	Lived.
93		136	0.92	39	Died, 47 days.
123		180	0.61	83	Lived.
135	178	176	0.69	64	Died, 59 days.
65		90	0.54	26	" 46 "
48.5		74	0.54	20	" 47 "
58.5		83	0.51	25	" 48 "
84		108	0.50	36	" 48 "
e. 98.8		137.7	0.66	50	

maximum weights in this table are given when they exceed the ghts.

ated from feed given, body weight of pig, and number of days.

Summary.

Feed.	Weight.				Deaths.
	Initial.	Final.	Daily gain.	Cottonseed meal eaten.	
	<i>lbs.</i>	<i>lbs.</i>	<i>lbs.</i>	<i>lbs.</i>	
No iron.	103.4	107	0.05	47	6
Copperas.	92.1	134.6	0.46	66	3
Ferric chloride.	102.2	163.2	0.68	80	1
Hardwood ashes.	98.3	137.7	0.67	50	7

ment 4.—A fourth experiment with iron salts was conducted under or conditions of the previous experiment. The pigs were of a or quality, which may account for the small gains made. Four even pigs each were fed on the following rations in the form of

- 42. Cottonseed meal 1 part plus cracked corn 7 parts,—control.
- 43. The same plus copperas solution.
- 44. The same plus iron-ammonium citrate solution.
- 45. Cottonseed meal 1 part, wheat bran 3 parts, cracked corn 4 parts.
- 46. Peanut meal 1 part and cracked corn 7 parts.

This experiment continued from November 29 to April 3 (127 days). There was one death in the control lot (Feed 342) in the 1st week and two in the copperas lot (Feed 343) on the 74th day and the 124th day. Disregarding these three pigs the average gains were best in the following order: (1) Iron-ammonium citrate. (2) Copperas. (3) Peanut meal. (4) Straight cottonseed meal. (5) Wheat bran.

TABLE V.

Feed.		Average weight.			Average gain.
		Initial.	Final.	Daily gain.	
342	Check.	55.5 (6 pigs.)	86.7	0.25	31.2
343	Copperas.	50.0 (5 ")	95.4	0.36	45.4 (42.1)*
344	FeCl ₃ .	50.7	105.1	0.43	54.4
345	Wheat bran.	52.8	90.8	0.30	38.0
346	Peanut meal control.	51.6	92.0.	0.31	40.3

* Including the gain of the two pigs that died.

All these pigs were in extremely poor condition at the end of the experiment. The gains were slight and the animals were found to be badly infested with internal and external parasites. Postmortem examination showed that practically all the animals were in a pathological condition.

Several experiments conducted at other stations have also indicated that iron salts have an antidotal action, and private individuals have also reported a beneficial action by copperas.⁴ It is apparent that where our experiments were conducted under very unfavorable conditions, the salutary effect of the iron salts is not so pronounced as under favorable conditions.

SUMMARY AND CONCLUSIONS.

1. Four feeding experiments with pigs have shown that iron salts have a decidedly beneficial action in preventing cottonseed meal injury. Much larger quantities of meal are consumed, deaths have been postponed or averted, and better gains have been made when an iron salt is added to the feed.

⁴ Walker, G. B., *Mississippi Agric. Exp. Station Bull.* 177, 1916. Wells, C. A., and Ewing, P. V., *Georgia Agric. Exp. Station Bull.* 119, 1916; also several personal communications.

2. Wood ashes apparently have no antidotal action in averting death but as the lot receiving ashes made much better gains it is possible that this is due to improvement of the inorganic part of the diet composed of corn and cottonseed meal.

3. The suggestion is made that the iron salts combine with, or facilitate oxidation of the harmful substances in cottonseed meal.

4. Iron salts have an antidotal action towards cottonseed meal poisoning of rabbits and swine.

5. By thus controlling the toxic factor, it is shown that cottonseed meal injury is not due to a lack of "vitamines" or to deficiencies in calcium, sodium, and chlorine,—which ash analyses might lead one to suspect as the limiting mineral factors in a diet of cottonseed meal and corn.

SOME FACTORS INFLUENCING THE RESPIRATION OF GROUND NERVOUS TISSUE.

By C. G. MACARTHUR AND O. C. JONES.

*(From the Department of Biochemistry of the University of Illinois, Urbana,
and the Department of Pharmacology of Stanford University
Medical School, San Francisco.)*

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We have undertaken to make a systematic study of respiration of the central nervous system. Distilled water, in spite of several disadvantages, was chosen as the medium, because by its use the smallest number of unknown influences are introduced. Physiological salt solutions did not give larger values than water. 1 per cent glucose, 1 per cent peptone, or 1 per cent lecithin solutions each gave less respiration than water. Blood as a medium for brain gives larger values than water or physiological salt solutions. But these larger values are partly due to the respiration of the blood itself, probably partly also to the catalytic action of the hemoglobin. The amount of oxidation in a slightly alkaline solution¹ is considerably larger than in water; but it is well known that dilute alkali is favorable for the oxidation of many tissue substances not necessarily related to respiration.

This report attempts to answer the following questions: Can a satisfactory extract of respiring substances be obtained? Does carbon dioxide or oxygen storage affect values obtained? Were the results altered by bacterial action? What is the temperature coefficient of that part of the respiration that is comparatively stable in water medium? Do blood constituents materially alter the amount of respiration obtained? How soon after the death of an animal does the rate become constant? How does brain respiration compare with that of other more carefully studied tissues in the same animal? Are there differences in the rate of oxidation in various divisions of the central nervous system? Are

¹ Batelli, F., and Stern, L., *Biochem. Z.*, 1909, xxi, 487.

there consistent differences in white and gray matter? Does the age of the brain affect the respiration in the same way as age affects the total metabolism of the animal? And, finally, how do various adult animals differ from each other?

The method used was similar to one that had given rather satisfactory results in a study of the rate of oxidation of several easily oxidizable compounds.²

Apparatus.

The *small tube* used in the following experiments is the Bunsel oxidase tube.³ It consists of an inverted Y-shaped test-tube with a ground glass stopper carrying a capillary manometer graduated in millimeters. The tube holds about 25 cc. When used in this investigation a 3 cc. test-tube containing 1 cc. of 0.5 N sodium hydroxide was inserted in the upper part after the tissue mixture had been introduced.

When ready for an experiment the small tubes were placed in a shallow perforated wooden box and clamped to it. This box holds eighteen small tubes. It was fastened to a mechanical shaker run by an electric motor. The shaker is in an air thermostat regulated to about 0.2°C.

Data for Calculation of Results.

The small tubes held 25 cc. In these there were placed 3 gm. of tissue, 6 cc. of H₂O, and 1 cc. of 0.5 N NaOH (in the inner 3 cc. test-tube). There were, therefore, approximately 15 cc. of space to be occupied by air, of which about 3 cc. would be oxygen. The atmospheric pressure averaged 750 mm. of mercury. 1 cc. of the gas in the tube corresponds to 50 mm. of mercury, and 1 mm. of mercury in manometer tubes represents 0.02 cc. of oxygen or 0.000024 gm. 0.02 cc. at 37° becomes 0.018 cc. at 0°.

The 1 cc. of 0.5 N NaOH was carefully measured with a pipette. At the end of the experiment the carbon dioxide present was determined by titrating the contents of the 3 cc. tube with 0.02

² Mathews, A. P., *J. Biol. Chem.*, 1909, vi, 3. MacArthur, C. G., *J. Physic. Chem.*, 1916, xx, 545.

³ Bunsel, H. H., *J. Biol. Chem.*, 1914, xvii, 409.

N H_2SO_4 using phenolphthalein as an indicator first, and then titrating to end-point with Congo red. The number of cc. taken to neutralize the solution to the Congo red end-point after neutralization to phenolphthalein approximately represents the amount of carbon dioxide present. 1 cc. of an hypothetical 0.02 N carbon dioxide solution would have 0.00044 gm., or 0.223 cc. of CO_2 in it. The number of cc. used for the Congo red titration as indicated above, multiplied by 0.00044 gives the weight of CO_2 liberated from the tissue in the given period of time; or if multiplied by 0.223, it gives the volume of CO_2 at 0° ; and if by 0.253, the volume at 37° .

In the following tables the figures in the last two columns are corrected to 0°C . and 760 mm. of mercury.

EXPERIMENTAL.

Tissue and Medium.—At first it was thought possible to use a solution of the respiring substances. If this could be done the technique would be greatly simplified and several sources of error would be eliminated. Centrifuging a water extract of the ground brain was tried repeatedly. The nature of the extracting medium was altered, also the fineness of grinding the tissue, the length of extracting time, and the length of time the water tissue was centrifuged. In all cases no distinct separation of the respiring substance could be obtained.

It was found that only a part of the respiring compounds go into solution. The amount varied considerably in the various experiments, depending on the length of time centrifuged and the time the tissue stood before centrifuging. It is entirely possible, that the rough separation of about two-thirds in the residue and one-third in the solution may indicate the comparative amounts of oxydones and oxidases present.⁴

Filtering through several layers of cheese-cloth gives milky solutions of widely different oxidative power. Filtering through paper was so slow and so incomplete and the changes in the medium were so large that it was deemed wholly unsatisfactory.

Because of unsatisfactory results with the centrifuging of the original hashed material, grinding the tissue with sand, glass, or

⁴ Batelli and Stern, *Compt. rend. Soc. biol.*, 1913, lxxiv, 212.

Kieselguhr and centrifuging the water extract were tried. It was found that if the grinding was thorough but little respiration occurred.⁵ This may be due to the destruction of the cells, or the sand may absorb one or more of the respiring substances, or the disintegration may allow cell localized substances to interact to prevent respiration.

Because of this lowering of respiration due to grinding the brain tissue in sand, it was of course necessary to show that the grinding with the meat grinder did not materially affect the results. In several experiments with tissue varying in size from that of the grinder (small sized grating used) to about one-fourth inch cubes, practically no differences in either oxygen consumed or carbon dioxide produced were found. It was therefore decided to use in this work the tissue ground with an ordinary meat grinder. However, the possibility of separating the compounds causing respiration is a very interesting one and is being studied more in detail.

O and CO₂ Storage.—It would increase the reliability of the results if it were known that the results obtained represented the amount of O used and CO₂ produced during the time of the experiment, and were not partially due to O and CO₂ in the tissue at the beginning of the experiment. To obtain some information about this point 20 per cent H₂SO₄ was used as a medium for the tissue in comparison with a water medium. It was hoped that this strong acid would stop the respiration and also drive out all the O and CO₂ in the tissue. It is believed that the results indicate this to be true.

TABLE I.
Calf Brain. O Reading 2 Hrs. after Death.

Time in hrs.....	Hg. by manometer readings.			Total O consumed.	0.02 N alkali used by CO ₂ .	O per gm. per hr.	CO ₂ per gm. per hr.
	1	2	3				
	mm.	mm.	mm.	cc.	cc.	cc.	cc.
Tissue in H ₂ O.....	16	28	38	0.76	2.3	0.072	0.048
“ “ “.....	17	30	40	0.80	2.5	0.076	0.053
“ “ H ₂ SO ₄	6	9	10	0.20	0.5	0.019	0.010
“ “ “.....	5	7	8	0.16	0.4	0.015	0.008

⁵ Harden, A., and Maclean, H., *J. Physiol.*, 1911-12, xliii, 34. Batelli and Stern, *Compt. rend. Soc. biol.*, 1914, lxxvi, 575.

Roughly speaking the amount of oxygen and carbon dioxide found in the tissues is between 15 and 20 per cent, of the total after 3 hours' shaking. It is possible, of course, that there is some respiration even in the presence of the strong acid. This is probably not large at any rate, because the results indicate that nearly all the oxygen consumed takes place in the 1st hour, whereas in respiration it continues throughout the experiment.

Two other flasks containing tissue and water were run along with the one in the above table. After the experiment strong H_2SO_4 was added to these flasks and shaken an hour longer to see how much CO_2 was left in the tissue after the 3 hour period. About the same amount remained in the tissue after the experiment as was found in the tissue at the beginning. Though the data do not give conclusive evidence, there seemed to be no reason for correcting the values directly obtained, either for oxygen or carbon dioxide.⁶

Bacterial Action.—Of course it was necessary to consider the possibility that the results were all due to bacterial action. There are several reasons for not believing so. First, though there is considerable variation in check experiments or in successive experiments, there is much greater uniformity and predictability than there would be were bacteria the cause. Second, it is well known that for some time following death bacteria get a foothold on a tissue with considerable difficulty, indicating protective substances. Third, if bacteria were the cause, one would expect that there would be much more oxygen consumed and carbon dioxide produced the last hour than the 1st. From the data it will be seen that as a rule there is a little more respiration the 1st hour. Lastly, it was found experimentally that following the orthodox aseptic practice in removing the brain, treatment of instruments, apparatus, etc., brought practically the same results as our regular technique. We obtained results after allowing the tissue to stand 8 hours in the ice box and then experimenting with it at 37° for 3 hours which showed that bacterial action had been very appreciable. It is believed that under the conditions of experimentation when the tissue is placed in tubes within 2 hours

⁶ Harden and Maclean, *J. Physiol.*, 1911-12, xliii, 34.

and is not kept at 37° more than 3 hours practically no bacterial action occurs.⁷

Effect of Heating to 100°.—It was also desirable to know whether heat stopped the respiration. The effect of boiling 3 gm. of the tissue plus the 6 cc. of water to be used in the tubes was the only heat factor studied.

TABLE II.

	Hg. by manometer readings.			Total O consumed.	0.02 N alkali used by CO ₂ .	O per gm. per hr.	CO ₂ per gm. per hr.
Time in hrs.....	1	2	3				
Old pig brain. O reading 3 hrs. after death.							
	mm.	mm.	mm.	cc.	cc.	cc.	cc.
Normal.....	8	18	25	0.50	3.1	0.048	0.065
Boiled 10 min.....	3	6	9	0.18	1.5	0.017	0.032
Old dog brain. O reading 3 hrs. after death.							
	mm.	mm.	mm.	cc.	cc.	cc.	cc.
Normal.....	15	30	40	0.80	3.5	0.076	0.073
Boiled 10 min.....	8	15	24	0.48	1.5	0.046	0.032

Though there is a rather wide variation in the results, it is clear that heating does not completely destroy either the oxygen consumption or the carbon dioxide production. Between a third and a half of the original respiration continues after thorough heating. This would indicate that this part of the respiration is different from the rest and that the substances producing it are more stable, not of an enzymic character. The other half is more unstable and resembles, to a certain extent at least, enzymes.¹

Toluene produced almost identically the same results as heat. It seemed probable that toluene altered the same substances in the same way as heat. Because of the susceptibility of the respiration to such substances they could not be used to preserve the tissue.

Temperature Coefficient.—With an increase of 10° in temperature there should be approximately a doubling in rate of reaction, if the process is a chemical one.

The following experiments at 37° were run in the air thermostat above mentioned. For those at 25° a water thermostat was used in which a brass mechanical shaker was operated at the same rate

⁷ Tashiro, S., *Am. J. Physiol.*, 1913, xxxii, 117.

as the one in the air thermostat. In the water thermostat the tubes up to the manometer portion were continually immersed in water. The reason for using both shakers was that the two temperatures could be compared on the same sample of tissue.

TABLE III.

	Hg. by manometer readings.				Total O con- sumed.	0.02 N alkali used by CO ₂ .	O per gm. per hr.	CO ₂ per gm. per hr.
Time in hrs.....	†	1	2	3				
Old pig brain. O reading 3 hrs. after death.								
°C	mm.	mm.	mm.	mm.	cc.	cc.	cc.	cc.
37.....	10	15	22	33	0.66	3.2	0.063	0.067
	8	12	18	28	0.56	3.1	0.053	0.065
	7	13	20	30	0.60	3.3	0.057	0.069
25.....	5	8	12	16	0.32	1.7	0.031	0.036
	4	8	12	16	0.32	1.5	0.031	0.032
Old dog brain. O reading 50 min. after death.								
37.....	11	20	32	46	0.92	3.5	0.087	0.074
	11	20	34	48	0.96	3.4	0.091	0.071
	13	22	36	50	1.00	3.6	0.095	0.076
25.....	6	10	15	30	0.60	2.0	0.057	0.042
	4	7	12	27	0.54	1.7	0.051	0.036
	4	8	13	28	0.56	1.9	0.053	0.040

If one compares the amount of oxygen consumed at the end of an hour it will be seen that the temperature coefficient is about 2.2, while at the end of 3 hours it is not far from 1.8. The temperature coefficient for carbon dioxide production is about 1.8.

There is very little doubt that the comparatively low temperature coefficient is correlated with the nature of the respiration in nerve and brain. At present it is difficult to say what kind of an oxidation would have so low a temperature coefficient.⁸ It may be that the explanation does not depend on a different kind of oxidation, but on the presence of temperature control substances that affect the rate more at higher temperatures than at lower.

Effect of the Blood in Tissue.—Though the portions of the brain used were freed carefully from blood, there was a very small amount present. To see what the rate of clot-free blood was

⁸ Maxwell, S. S., *J. Biol. Chem.*, 1907, iii, 359.

and to see whether it had an accelerating action or simply an additive effect on tissue, the following experiments were undertaken. 6 gm. of clot-free blood plus 3 cc. of H_2O were used in one set of tubes; in the blood and brain set 6 gm. of blood and 3 of brain were used.

TABLE IV.
Sheep Brain. O Reading 1½ Hrs. after Death.

Time in hrs.....	Hg. by manometer readings.				Total O con- sumed.	0.02 N alkali used by CO ₂ .	O per gm. per hr.	CO ₂ per gm. per hr.
	½	1	2	3				
	mm.	mm.	mm.	mm.	cc.	cc.	cc.	cc.
Normal tissue.....	11	19	42	57	1.14	4.2	1.08	0.088
“ “	11	20	42	60	1.20	4.1	1.14	0.086
Blood.....	2	5	8	20	0.40	2.4	0.038	0.050
“	2	5	7	18	0.36	2.0	0.034	0.042
Blood and brain.....	15	30	50	72	1.44	5.5	0.136	0.115
“ “ “	16	35	52	75	1.50	5.7	0.142	0.120

Other experiments substantiate the above results, indicating rather strongly that the effect of blood is largely additive. Therefore the small amount of blood present in the brain used would produce a very negligible effect on the respiration.

Effect of Standing on Respiration.—Another point that needed to be studied was the effect on the oxygen consumption and carbon dioxide production of allowing the tissue to stand.

In the following experiment an old dog was killed, the brain removed and ground, 3 gm. with 6 cc. of water were placed in the tubes, and the small test-tube containing 1 cc. of 0.5 N NaOH was inserted. In the experiment recorded below this occupied 12 minutes. After the tubes were placed in the thermostat 10 minutes were allowed for the contents to come to the temperature of the thermostat. This length of time had previously been found to be sufficient if the tissue, which remained nearly the temperature of the animal, was added to water that had been heated to 37°C. Thus the O reading on the first set of tubes was obtained 22 minutes after the death of the animal. The second set was started 1 hour later than the first, the third set 3 hours after the first, and the last set 5 hours after. Up to the time of its use for the experiment the tissue was kept in the ice box.

TABLE V.
Old Dog Brain. O Reading 22 Min. after Death.

Time in hrs.	Hg. by manometer readings. .				Total O con- sumed.	0.02 N alkali used by CO ₂ .	O per gm. per hr.	CO ₂ per gm. per hr.
	†	1	2	3				
	mm.	mm.	mm.	mm.	cc.	cc.	cc.	cc.
0.	8	16	28	38	0.76	3.2	0.072	0.067
	9	16	28	38	0.76	3.5	0.072	0.074
1.	5	14	22	28	0.56	2.8	0.053	0.059
	4	10	28	(30)	0.60	2.5	0.057	0.053
3.	4	14	24	32	0.64	2.5	0.061	0.053
	5	16	24	29	0.58	2.5	0.055	0.053
5.	3	9	17	28	0.56	2.5	0.053	0.053
	4	8	17	29	0.58	2.4	0.055	0.051

There seems to be considerably less respiration in the tissue after an hour of standing in the ice box than there is soon after death. The data would indicate that there is a rather rapid decrease in the respiration following death, but that it soon becomes nearly constant probably not far below the normal rate. After the preliminary rapid falling off in rate of oxygen consumption the tissue respiration lowers but slowly for several hours, 5 in this experiment, and 7 in another that gave very similar results in every way.⁷

Not only in the above data but in others to be presented it will be noticed that there is a rather large variation in check experiments. This variability may be due to a combination of factors. Very likely the 3 gm. portions used did not contain the same number of pieces of brain of the same size; in another experiment it was shown that while this factor did not make a great difference, the difference was appreciable. Another factor entering into the above variations is the thoroughness with which the water present was mixed with the tissue. Though every tube was treated as nearly as possible alike, there were surely small differences. The weight of the tissue used (3 gm.) varied considerably. This was probably a large factor in producing the variations in the above table. The tissue was weighed on small horn pan balances. It was necessary to weigh quickly to avoid effect of air, and especially to avoid the effect of standing on the rate of respiration.

Comparison of Brain Respiration with That of Other Tissues.—For the sake of comparison, and because so much more work has

been done on other tissues, the following experiments were carried out on brain, liver, kidney, and muscle. 3 gm. portions of these organs from guinea pigs were run in as nearly as possible the same way at the same time, to show what were the differences in oxygen consumption and carbon dioxide production.

TABLE VI.

Time in hrs.....	Hg. by manometer readings.				Total O con- sumed.	0.02 N alkali used by CO ₂ .	O per gm. per hr.	CO ₂ per gm. per hr.
	1	1	2	3				
Old guinea pig. O reading 40 min. after death.								
Brain.....	13	17	30	44	0.88	3.6	0.074	0.076
Liver.....	10	20	42	62	1.24	3.8	0.118	0.080
Kidney.....	8	14	28	44	0.88	3.4	0.074	0.071
Muscle.....	6	10	20	31	0.62	2.7	0.059	0.057
Younger guinea pig. O reading 1 hr. after death.								
Brain.....	12	20	50	72	1.44	4.4	0.137	0.093
Liver.....	13	24	58	78	1.56	4.6	0.148	0.097
Muscle.....	8	14	26	42	0.84	2.6	0.080	0.055
Old guinea pig. O reading 40 min. after death.								
Brain.....	9	16	32	44	0.88	4.1	0.084	0.086
Liver.....	15	26	48	64	1.28	4.9	0.122	0.103
Kidney.....	10	20	40	52	1.04	4.1	0.099	0.086
Muscle.....	7	10	20	32	0.64	3.1	0.061	0.065

The rate of oxidation of ground brain tissue 1 to 3 hours after death is not quite so great as that of liver from the same animal treated in the same way. It has about the same rate as the kidney and is considerably larger than that of the muscles. If there is a primary respiration occurring in the living intact cells that rapidly disappears in the treatment used in these experiments, the data given furnish a comparison of the secondary respiration only.

It will be noticed that the oxidation in brain tissue does not increase as rapidly during the 2nd and 3rd hours as does that for the other tissues. May this be connected with the fact that autolysis of brain is so much slower than that of other tissues? There seems to be more protective material to keep the brain in its original condition than in other tissues.

ons of the Nervous System.—The question that was most worked out was the comparison of the respiration of the parts of the brain. Samples from various parts of the a single animal were treated in exactly the same way and 1 at the same time.

TABLE VII.

	Hg. by manometer readings.				Total O consumed.	0.02 N alkali used by CO ₂ .	O per gm. per hr.	CO ₂ per gm. per hr.
	$\frac{1}{2}$	1	2	3				
6 mos. old calf. O reading 1 hr. 50 min. after death.								
	mm.	mm.	mm.	mm.	cc.	cc.	cc.	cc.
l.....	8	16	40	65	1.30	4.1	0.123	0.086
m.....	9	17	45	70	1.40	4.1	0.133	0.086
	7	16	38	60	1.20	4.0	0.114	0.084
	8	12	32	50	1.00	3.6	0.095	0.076
allosum....	5	10	30	45	0.90	3.6	0.086	0.076
rd.....	5	10	26	45	0.90	3.4	0.086	0.071
erve.....	5	10	25	42	0.84	4.7	0.080	0.099
Old beef. O reading 2 hrs. 15 min. after death.								
	mm.	mm.	mm.	mm.	cc.	cc.	cc.	cc.
l.....	7	15	30	45	0.90	3.3	0.086	0.069
	8	14	26	40	0.80	3.4	0.076	0.071
m.....	11	17	32	46	0.92	3.1	0.087	0.065
	10	15	28	44	0.88	3.1	0.084	0.065
	6	13	21	30	0.60	3.3	0.057	0.069
	7	15	24	36	0.72	3.3	0.068	0.069
allosum....	5	9	15	25	0.50	2.7	0.048	0.057
	6	11	18	27	0.54	2.7	0.052	0.057
rd.....	4	6	12	17	0.34	2.1	0.032	0.044
	5	7	12	16	0.32	2.0	0.030	0.044
1 yr. old calf. O reading 1 hr. 50 min. after death.								
	mm.	mm.	mm.	mm.	cc.	cc.	cc.	cc.
l.....	10	18	40	70	1.40	4.5	0.133	0.095
	10	19	38	68	1.36	4.4	0.129	0.092
m.....	10	17	40	66	1.32	4.6	0.125	0.097
	9	16	37	67	1.34	4.4	0.127	0.092
	5	10	24	46	0.92	3.0	0.087	0.063
	5	11	25	47	0.94	3.2	0.089	0.067
	5	10	20	32	0.64	2.8	0.061	0.059
	5	10	21	33	0.66	2.7	0.063	0.057
allosum....	7	14	28	50	1.00	3.2	0.095	0.067
	8	15	32	48	0.96	3.1	0.091	0.065
rd.....	4	7	15	26	0.52	2.7	0.050	0.057
	4	8	17	28	0.56	2.7	0.054	0.057

There is but little difference either in oxygen consumption or carbon dioxide production between the cerebrum and the cerebellum. The midbrain respire more slowly than these but not so slowly as the medulla, whose rate is about three-fourths of that of the cerebellum. Both the oxygen used and the carbon dioxide formed are still less in the corpus callosum, but the carbon dioxide is not generally as much less as is the oxygen. It will be noticed throughout these results that the more medullary sheath material there is present the higher the relative carbon dioxide production. This may indicate that a part at least of the respiration in nerve fiber is different from that in nerve cell. This is very apparent in the spinal cord, where the oxygen consumed is about half of that in the cerebrum, while the carbon dioxide produced is about two-thirds as much as in the cerebrum. Though there is but one experiment in which sciatic nerve is compared with the other divisions of the nervous system, the rate of oxygen consumption is even less than that of the spinal cord; but the carbon dioxide produced is even larger than that in the cerebrum. This may be an error, but is very interesting if true. This gives a respiratory quotient much larger than for the other portions of the nervous system.⁹

It would, of course, be premature to attempt to correlate in any way the data given with the degree of differentiation of the different brain divisions or with their biological functions.

Gray and White Matter.—Gray matter selected from the outside

TABLE VIII.
Calf Brain. O Reading 1½ Hrs. after Death.

Time in hrs.....	Hg. by manometer readings.			Total O consumed.	0.02 N alkali used by CO ₂ .	O per gm. per hr.	CO ₂ per gm. per hr.
	1	2	3				
	mm.	mm.	mm.	cc.	cc.	cc.	cc.
Gray.....	25	45	65	0.30	4.0	0.124	0.064
	24	42	62	0.24	4.2	0.118	0.088
	24	44	60	0.20	4.1	0.114	0.086
White.....	14	26	35	0.70	2.6	0.067	0.055
	15	25	34	0.68	2.8	0.065	0.059
	12	24	35	0.70	3.0	0.067	0.063

⁹ Tashiro, *Am. J. Physiol.*, 1913, xxxii, 130.

layer of the cortex was compared with white matter from the corpus callosum.

As was to be expected from the preceding tables, gray matter uses more oxygen and forms more carbon dioxide than white matter. There is almost twice as much oxygen used by the gray, but the white produces about two-thirds as much carbon dioxide as does the gray. The respiratory quotient is consequently higher in white matter than in gray. This table also emphasizes the fact that there is a higher respiratory quotient where the amount of medullation is greater.

Work now in progress on the autorespiration of cephalin, which occurs in much greater amount in white matter, may furnish a clue to this difference between white and gray matter. There is considerable indirect evidence also that the nucleoproteins are concerned with brain respiration.¹⁰ It would simplify matters greatly if it were found that a lipid was concerned with one form of respiration and a nucleoprotein with another.

Comparison of Ages of Animals.—It is to be expected that the younger the brain, the faster its metabolism. We know this to be true for the whole animal. This is supposed to be due to growth of cells, and a larger proportion of the more reactive sub-

TABLE IX.

Time in hrs.	Hg. by manometer readings.				Total O consumed.	0.02 N alkali used by CO ₂ .	O per gm. per hr.	CO ₂ per gm. per hr.
	‡	1	2	3				
Beef.								
	mm.	mm.	mm.	mm.	cc.	cc.	cc.	cc.
Cerebrum, 8 mos.	11	20	45	62	1.24	4.1	0.118	0.086
“ old.	7	15	30	45	0.90	3.2	0.086	0.067
Cerebellum, 8 mos.	10	20	45	60	1.20	4.0	0.114	0.084
“ old.	10	15	28	44	0.88	3.1	0.084	0.065
Dog.								
Whole brain, 3 mos.	13	23	40	57	1.14	3.8	0.108	0.080
“ “ fat and old.	10	18	30	42	0.84	2.9	0.080	0.061
“ “ adult.	9	16	28	38	0.76	3.2	0.074	0.067

¹⁰ McGregor, H. H., *J. Biol. Chem.*, 1916-17, xxviii, 403. Batelli and Stern, *Biochem. Z.*, 1911, xxxiv, 263. Lillie, R. S., *Am. J. Physiol.*, 1902, vii, 412.

stances in the cells, with a consequent smaller percentage of the less reactive colloids.¹¹

By consulting previous tables it will be seen that in each division of the brain the same decrease in rate of respiration occurs. What is true of respiration is undoubtedly true of its complement metabolism. With an increase in amount of oxidation, there must be an increase in amount of food substances oxidized, with a corresponding increase in metabolic products excreted. During growth the rate of oxidation decreases, but the complexity of the reaction brought about by the oxidation increases.

Comparison of Different Adult Animals.—Though the animals used in these experiments were adults they varied greatly in size, age, and nutritional condition. The figures in parentheses in the first column (Table X) give the number of experiments averaged to give the results in the table. The table is arranged in order of size of the animals.

TABLE X.
O Reading about 2 Hrs. after Death.

Time in hrs.....	Hg. by manometer readings.				Total O consumed. cc.	0.02 N alkali used by CO ₂ . cc.	O per gm. per hr. cc.	CO ₂ per gm. per hr. cc.
	1	2	3	4				
	mm.	mm.	mm.	mm.				
(3) Frog, brain and spinal cord.....	5	10	18	32	0.64	1.8	0.061	0.038
(3) Mouse.....	18	26	47	68	1.36	4.6	0.129	0.097
(2) Rat.....	10	21	38	57	1.14	4.4	0.108	0.092
(3) Guinea pig.....	10	18	35	58	1.16	4.3	0.110	0.090
(1) Cat.....	10	17	36	56	1.12	3.8	0.106	0.080
(2) Dog.....	9	17	32	44	0.88	3.3	0.084	0.069
(2) Sheep.....	8	15	28	48	0.96	3.6	0.091	0.076
(3) Pig.....	7	13	20	36	0.72	3.0	0.068	0.063
(3) Beef.....	7	15	30	45	0.90	3.2	0.086	0.067

The theory that the greater the surface area of an animal the greater will be its food and oxygen requirements is found to apply in a general way to brain respiration. In the brain, however, one would not expect so direct a relation between surface evaporation and rate of metabolism as in other parts of the body. It is interesting to note that the indolphenol oxidase content¹² of the

¹¹ Koch, W., and Koch, M. L., *J. Biol. Chem.*, 1913, xv, 423.

¹² Vernon, H. M., *J. Physiol.*, 1911-12, xliii, 96.

brains of animals varies with size in a similar way to that of respiration.

The frog brain and spinal cord do not seem to have as great a capacity for oxidation as those of the higher animals. On first thought one might expect that with evolution there would be greater oxidative efficiency. But a consideration of the data for mammals (though but suggestive because of lack of higher forms) indicates brain size as another factor in mental complexity. If rate of respiration were a measure of brain ability, the mouse should be capable of more complex activity than the dog or sheep; but because the brain of the dog or sheep is larger (thus having more nerve cells and more connections) their mental power, as well as their total brain oxidation, is greater. There is also reason for believing that the nature and amounts of the colloidal substances in nerve cells are important in determining the intensity and character of the response. Judging from other data as well as from those given in this paper, brain evolution, from both the phylogenetic and ontogenetic standpoints, seems to be accompanied by a decrease in the rate of chemical activity, but by an increase in the complexity of the colloidal substratum and an increase in the number of cells.

SUMMARY.

1. The order in which the various divisions of the nervous system respire was found to be the following: cerebrum, cerebellum, midbrain, medulla, corpus callosum, spinal cord, then nerve.
2. The smaller the animal the more rapidly, as a rule, its brain consumes oxygen and gives off carbon dioxide.
3. In an adult animal brain respiration is less than in a young one.
4. Gray matter consumes about twice as much oxygen as white matter, but about two-thirds as much carbon dioxide is produced by the latter.
5. Grinding brain tissue with sand destroys its power of auto-oxidation.
6. Heating to 100° or addition of toluene (probably anesthetics too) reduces the amount of respiration more than half, indicating a thermostabile and thermolabile factor.
7. The temperature coefficient for brain oxidation is about 1.8.

8. Ground guinea pig brain tissue oxidizes at about the same rate as kidney tissue, faster than muscle, but not so rapidly as liver. There is not, however, a large difference between them.

9. Blood in brain tissue may have a small catalyzing effect in very small quantities, but when appreciable amounts are present, its respiratory effect is but little more than additive.

THE DIRECT AND INDIRECT CALORIMETRY OF CASSIOPEA XAMACHANA.

THE EFFECT OF STRETCHING ON THE RATE OF THE NERVE IMPULSE.

By J. F. McCLENDON.

(From the Marine Laboratory of the Carnegie Institution of Washington
and the Physiological Laboratory of the University of
Minnesota, Minneapolis.)

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The object of the present paper is to throw light on the factors influencing the oxidation rate of living cells. Henze (1910, a) observed that sea anemones use less oxygen when less is present in the sea water, but interpreted this as due to the time required for diffusion into the animal. That is to say, he supposed that all of the cells were not supplied with oxygen when there was little in the sea water. If oxygen was entirely absent in some of the cells, the decreased oxidation may have been merely the expression of the lesser number of cells taking part in the metabolism. Burrows showed that tissue cells require a certain oxygen tension for growth, and Loeb and Wasteneys found that the heart beat of *Fundulus* embryos may be slowed by reducing the oxygen. The growth of *Fundulus* may be suspended by lack of oxygen and may be slowed by diminishing the oxygen.

EXPERIMENTAL.

The calorimeter consisted of either an 840 cc. or a 900 cc. Dewar flask (thermos bottle) enclosed in an air tight container, which was immersed in water that was maintained at the same temperature as the water in the flask, within 0.003°. The technical difficulties were met as follows: Two Beckmann thermometers were adjusted and compared over the range of temperatures of the experiments, and fitted with reading lenses to estimate down to 0.001°. A large tank of sea water was brought to 30° (which was about the temperature of the air) and its pH and O₂ and CO₂ content determined. Some of this sea water was dipped out and a *Cassiopea* in-

roduced into it. The thermos bottle, stopper, and thermometers were immersed in the large tank until they reached the temperature of the water. The *Cassiopea* was transferred to the thermos bottle and a perforated cork stopper inserted with the exclusion of air bubbles. One Beckmann thermometer was inserted through the perforation in the stopper and the other suspended in the tank, near the middle of the thermos bottle. A small hole, remaining in the stopper for exit of displaced water, was closed with wax. The pulsations of the *Cassiopea* stirred the water inside the thermos bottle; and the water in the tank was mechanically stirred and was kept at the same temperature as that inside the thermos bottle by additions of small portions of warmer or colder water as required. The light was excluded by the silvering and coverings of the thermos bottle, but in some experiments, in which a 900 cc. glass jar with ground glass cover was used in place of the thermos bottle, the light could be excluded by darkening the tank, so as to prevent photosynthesis in the symbiotic plant cells. Time was measured by means of a stop-watch and a clock. The same *Cassiopea* was used in a series of experiments.

The oxygen in the sea water was determined by the Winkler method, which can be corrected for the slight error due to a slight amount of organic matter given out by *Cassiopea*, and it was thought impracticable to use the complicated method of Shutzenberger and Risler (Hense, 1910, b). A 250 cc. glass-stoppered bottle was weighed empty and full of distilled water at a known temperature, in order to standardize its volume. It was fitted with a double-bored rubber stopper and a long and a short glass tube with rubber connections. The bottle was filled with mercury and the long glass tube sucked full of the water to be analyzed and the stopper inserted. By inverting the bottle, the sea water was siphoned into it, when the rubber stopper was removed and the glass stopper inserted. The glass stopper was lifted and 1 cc. of alkaline KI solution and 1 cc. of 40 per cent $MnCl_2$ solution introduced (correction being made for this in the O_2 calculation) and the stopper inserted. Colloidal membranes formed about the drops of alkali and violent shaking was necessary to break them. After the precipitate had settled, 2 cc. of concentrated HCl were added and the stopper inserted and the bottle shaken. Its contents were transferred to a flask and titrated with 0.01 N sodium thiosulfate solution until the yellow color disappeared, then starch solution was added and the titration continued until the blue color disappeared, then the water was poured into the bottle and back into the flask and titrated until the blue color disappeared. The calculation was as follows:

$$\frac{0.056 \times \text{cc. thiosulfate}}{\text{capacity of bottle} - 2} = \text{cc. of oxygen per liter of sea water, and then}$$

$$\text{quotient} \times 0.9 = \text{cc. of oxygen in calorimeter (on the assumption that the concentration of oxygen in the } Cassiopea \text{ was the same as that in sea water)}.$$

In order to reduce the carbonate content, the alkaline KI solution was made fresh every few days from two stock solutions. Carbonate-free NaOH solution was made by dissolving 100 gm. of NaOH in 100 cc. H_2O .

in a glass-stoppered bottle and pipetting off after the carbonate had settled. One part of this was mixed with one part of 20 per cent KI solution before being used. NaOH made from metallic sodium contained a trace of nitrite and NaOH purified by alcohol contained but little more. This nitrite causes no error if the titration is quickly made immediately after adding the acid, but the contents of the flask slowly turn blue for hours after the end-point has been reached. If the acidity is greatly increased, however, the nitrite causes an appreciable error in the titration.

The MnCl_2 solution contained a trace of $\text{Mn}(\text{OH})_2$, but this was removed by decantation. The thiosulfate was dissolved in CO_2 -free water and kept in an automatic burette with soda-lime tubes, and standardized with pure iodine. The starch solution was allowed to settle and only the clear upper portion used. When dextrins appeared in it, a fresh solution was prepared.

The number of gm. of chlorine per kilo of sea water (abbreviated Cl) was determined by titrating with a silver nitrate solution standardized with standard sea water from the International Commission. The alkaline reserve was determined by titrating 100 cc. of sea water with 0.01 N HCl, while boiling in a 500 cc. Erlenmeyer flask of resistance glass, using dibrom-*o*-cresolsulfophthalein as indicator, until every trace of purple color had permanently disappeared. The water must not dry on the sides of the flask. The pH was determined colorimetrically by means of the method and identical tubes previously described (McClendon, 1917, b). Correction was made for Cl. It was found that new supplies of indicators must be tested before being used to see that they have the proper pH range. The total CO_2 was determined from the pH and alkaline reserve by means of the conversion table (McClendon, 1917, b).

The weights and one thermometer were standardized by the United States Bureau of Standards and the other apparatus was standardized with them.

Although pH and CO_2 determinations were made in all experiments, it was found that the oxygen absorbed could be determined much more accurately than the CO_2 given out, and the CO_2 determinations are not listed with the experiments, but are collected together in the form of respiratory quotients. The respiratory quotients were 0.7, 0.74, 0.76, 0.84, 0.85, 0.86, 0.88, 0.9, 0.91, 0.92, 0.97, 0.99, 1, 1.02, 1.03, 1.1, 1.15, 1.2. It was impossible to tell whether this variation is due entirely to errors in the CO_2 determinations or whether the respiratory quotient varied. It is improbable, however, that respiratory quotients of 1.2 existed for even short periods of time, and these at least may be considered due to technical errors. According to Mayer (1914) *Cassiopea* lives on animal food exclusively and does not

absorb carbohydrates from its symbiotic algæ, since it starves as rapidly in the light as in the dark. It may, however, get some carbohydrates from its animal food, or from glycoproteins during starvation. In this connection it may be of interest to note that *Cassiopea* secreted a mucin-like substance. Since the error in estimating CO_2 production may be 30 per cent in half hour experiments with small *Cassiopeas*, it is convenient to assume that the respiratory quotient is constant and is about 0.95, which is also the average found by Vernon for the hardier species of jellyfish on which he made most of his determinations.

The oxygen consumption is about doubled when the temperature is raised from 20° to 30° , and from results on other animals is probably an exponential function of the difference in temperature. Harvey (Mayer, 1917) found the velocity of the nerve impulse in *Cassiopea* to be a linear function of the temperature, and to increase about 64 per cent on raising the temperature from 23° to 33° . I found the activity of the ganglia (rhopalia) in inducing pulsations of the umbrella to be about doubled with rise from 20° to 30° in temperature. The data were as follows:

Cc. O_2 consumption.		Temperature coefficient (or 10° rise.	Pulsations per sec.	
20°	30°		20°	30°
0.698	1.54	2.21		
0.57	1.05	1.84	0.27	0.55

These experiments show the necessity of accurate temperature control and in all of the other experiments in this paper the temperature was measured to the nearest tenth of a degree and maintained to within 0.2° of 30° .

The sea surface is at about the optimum pH for metabolism (usually 8.1–8.3), but the variation in metabolism with variation of pH within the range studied is very slight, as shown by the following table (diameter of *Cassiopea* = 10 cm.).

pH	O ₂ concentration.	O ₂ used.
7.50	4.40	1.85
7.53	4.50	1.95
8.24	4.30	2.79
8.24	4.50	2.87
8.24	4.55	2.43
8.38	4.40	2.07
8.52	3.90	2.28
8.72	3.16	2.59

These variations in oxidation may be due to experimental errors and variation in O₂ concentration, except the first two which show a slight lowering of oxidation when the pH is reduced to about 7.5.

In comparing the rise in temperature in the calorimeter with that calculated from the O₂ consumption, the assumptions were made that the respiratory quotient was 0.95, and that a mixture of proteins, fats, and carbohydrates was burned, giving 6 gm. calories per cc. CO₂, as in the following table.

O ₂	CO ₂	Gm. calories.	
		Determined.	Calculated.
2.18	2.07	12.6	12.4
2.54	2.41	16.2	14.5

In these experiments it was assumed that there was no loss of heat, although some heat must have passed into the thermometer bulb and glass lining of the thermos bottle. The specific heat of the sea water and *Cassiopea* was taken as unity because the determinations were not accurate enough to warrant the application of small corrections. The experiments had to continue for 2 hours in order to obtain an accurately measurable rise in temperature, and the tedium of keeping the tank at the same temperature as the calorimeter necessitated the substitution of indirect calorimetry in the remainder of the experiments.

Some preliminary experiments to show the effect of O₂ concentration are as follows:

Average O ₂ concentration.....	11.9	1.91	3.36	1.55
O ₂ consumption.....	2.6	2.38	2.26	1.65

In performing these experiments, a number of possible sources of error were thought of and it was decided to make a more detailed study of the metabolism of *Cassiopea* before returning to the subject. The chief danger of error was in prolonging the experiment until all of the O₂ was used up. It was found that *Cassiopea* could live more than 7 hours without oxygen, in which case no measurable quantity of CO₂ was produced. Vernon observed practically no increase in the respiratory quotient of jellyfish correlated with oxygen-want, whereas the respiratory quotient of fishes increased under these conditions.

In order to determine whether the rate of oxidation depends on the oxygen concentration it is desirable to know something about the oxygen concentration inside the living cells. In other words, the transfer of oxygen to the cells must be facilitated as much as possible if we are to judge anything about the concentration of O₂ within them from that in the sea water. This could be approximated by agitating free cells or a single layer of cells with the water or circulating the water over a single layer of cells. When using free cells, some are liable to injury and more or less disintegration, thus interfering with the titrations, but notwithstanding the criticism of Heilbrunn, comparative results may be obtained (McClendon and Mitchell). *Cassiopea* was chosen, because the cells are spread in thin layers on the surface of a mesoglea which will be shown to use practically no oxygen. The pulsations of the *Cassiopea* (Mayer, 1906) bring currents of water over the cell layers, so that diffusion is necessary only for a minute distance. The error due to this diffusion would be large only when the O₂ concentration is very small. By skilful manipulation, the mucous secretion may be prevented from increasing or leaving the surface of the *Cassiopea*.

Evidence that oxidation is confined to the cell layers is apparent in the fact that oxidation is not proportional to the volume but to the surface. It would be practically impossible to measure the surface, but since the individuals are practically of the same shape, the surface is proportional to the square of

the diameter. Since *Cassiopea* is elastic, the diameter was always measured under the same conditions; i.e., resting on a glass plate, with the exumbrella in contact with the glass (and the average of two diameters at right angles to one another taken). Some rough preliminary determinations showed the O₂ consumption in cc. per hour to be about 0.023 × the square of the diameter in cc. as shown in the following table.

Diameter in cm.....	3.5	7	8.5	9.5	10	11.5
O ₂ per hr.....	0.4	1.4	1.17	2.16	2.7	2.78

Very small *Cassiopeas* used more O₂ than calculated from the formula (an anomaly which is correlated with more rapid pulsations). A *Cassiopea* 3.5 cm. in diameter pulsated once a second whereas one 10 cm. in diameter pulsated 0.3 times per second. In order to compare experiments on *Cassiopea* where the weight is recorded, it is convenient to know that the diameter in cm. = $2.25 \times \sqrt[3]{\text{weight in gm.}}$

The following table gives the respiration rate under different conditions, except that the temperature is always 30°.

Diameter.	O ₂ per hr. square of diameter	O ₂ per liter.	pH	O ₂ per hr.
cm.				
7	0.0200	4.5	8.20	1.3
8	0.0187	4.2	8.22	1.26
8.5	0.0152	3.32	8.22	1.09
10	0.0287	4.5	8.24	2.87
10	0.0279	4.3	8.24	2.79
10	0.0243	4.55	8.24	2.43
10	0.0185	4.4	7.50	1.85
10	0.0195	4.5	7.53	1.95
10	0.0207	4.4	8.38	2.07
10	0.0228	3.9	8.52	2.28
10	0.0259	3.16	8.72	2.59
11	0.0230	3.54	8.17	2.79
11	0.0200	1.8	8.40	2.43
11	0.0160	1.57	8.02	1.93
11	0.0320	7.2	8.47	3.84
11	0.0400	7.44	8.46	4.87

In the above table the pH and O₂ per liter at the beginning of the experiment are given and the pH was about 0.09 and O₂ 1.5 cc. lower at the end of each experiment. The average O₂ during each experiment influences the O₂ used per hour, but apparently no difference in the quotient of the O₂ used per hour divided by the square of the diameter (second column) can be correlated with difference in size. Using greater extremes of size, however, the quotient seems to decrease as the diameter increases, and therefore extreme sizes were usually avoided.

All of the experiments were made under conditions of starvation and hence the *Cassiopea* used its own substance as a source of energy. Starvation can hardly be considered a pathological process in *Cassiopea*, however, since it may remain alive for months without food, constantly decreasing in weight until it almost disappears before death. Mayer (1914) determined the loss in weight as about 5.6 per cent per day at about 30°, although no thermostat was used. If y is the weight at any moment and w is the weight when starvation commenced and n is the number of days of starvation,

$$y = w (1 - 0.056)^n$$

Since I found the diameter to be 2.25 times the cube root of the weight, if the weight were 100 gm., the diameter would be 10.45 cm. The O₂ consumed during 1 day would be about 0.023 times the square of the diameter times 24 = about 60 cc. O₂ absorbed and 57 cc. CO₂ given out. If we assume that protein was burned and that 5.9 gm. calories correspond to 1 cc. CO₂, the metabolism would equal 336 gm. calories for the day. If we assume a certain mixture of proteins, fats, and carbohydrates were burned and 6 calories correspond to 1 cc. CO₂, the metabolism would equal 342 calories per day.

Since I have shown that the metabolism is proportional to the surface and Mayer has shown that the loss in weight is proportional to the volume (weight), the composition of the *Cassiopea* must change during starvation. In other words, it loses weight faster than it burns protein (or other organic matter), and hence the concentration of the protein must increase. Mayer (1914) found the cellular layer did not decrease in thickness during starvation, and Hatai found the percentage of ni-

trogen to the total body weight increases during starvation, and is also greater in small than in large, well nourished *Cassiopeas*. Therefore in attempting to calculate the metabolism from the loss in body substance, we should make it proportional to the loss in surface rather than loss in volume, since the loss in living matter seems to be proportional to the loss in surface, and the *Cassiopea* seems to have no other important store of food than its own protoplasm, the mesoglea apparently functioning chiefly as a skeleton. Since the surface is proportional to the two-thirds power of the volume, we may assume that the protein is proportional to the two-thirds power of the weight (the density remaining practically constant). The protein equals 5.16 per cent of the two-thirds power of the weight (calculated from Hatai's data on the assumption that protein is 16 per cent N). The weight at the beginning of starvation was 100 gm. and the protein 1.107 gm.; at the end of 1 day the weight was 94.4 and the protein 1.07, being a loss of 37 mg. of protein. If we assume that 1 mg. of protein is equivalent to 4.4 calories the metabolism the 1st day would be 163 calories, although I found it to be 336-342 calories. Although these calculations are only approximate, since starvation is a little greater the 1st day than calculated by the formula, this great difference indicates that the burning of protein does not account for all of the heat. Since living cells contain lipoids or lipo-proteins, and carbohydrates or glycoproteins, it seems probable that proteins, carbohydrates, and lipoids are burned. The mesoglea has not been analyzed separately, but is largely sea water, with possibly a trace of glycoprotein. It probably has little calorific value, since the use of a store of food would cause a relatively greater metabolism in large starving *Cassiopeas* than was actually observed.

Since the lining of the alimentary tract is not at the surface of the *Cassiopea* and O_2 must diffuse through at least a mm. of tissue to get to it, it was decided to pull off the manubrium and study the metabolism of the umbrella. The umbrella is disc-shaped and covered on both sides by epithelium, and pulsates, thus circulating the water. The wound made by removal of the manubrium is of small area and is covered by an epithelium within a few hours, and the umbrella will live as long as a starving *Cassiopea*. Some rough determinations indicate

that the respiration of the umbrella is only about a fourth as great as that of the whole *Cassiopea*. In the following table are recorded measurements on three *Cassiopeas* and on their umbrellas after removal.

Diameter.	O ₂ per hr. square of diameter		Pulsations per sec.
	<i>Cassiopea</i> .	Umbrella.	
cm.			
3.5	0.033	0.0075	0.94
9.5	0.021	0.0041	0.58
11.5	0.030	0.0075	0.63

Since the respiration is influenced by the muscular activity or pulsation rate and the latter is not constant, it was decided to remove the ganglia (rhopalia) that induce the pulsations and start a continuous contraction wave running around the subumbrella, the middle third of which has no neuromuscular tissue (Mayer, 1908). The rhopalia were cut out by means of a cork borer and the wave was started by electrical stimulation. It was noticed, however, that the contraction wave, apparently constant for short intervals of time changed more rapidly at first and then more slowly, but never became absolutely constant, the change being perhaps associated with shrinkage of the umbrella. The effect of shortening and stretching on the contraction wave was therefore studied. The rate of the contraction wave depends on the rate of the nerve impulse around the circuit of the nerve-muscle layer, but does not depend solely on the rate in the neuraxon, since there are numerous synapses, and furthermore, the path of the impulse is zigzag. Professor Cary kindly showed me a stained preparation of the nervous network of the subumbrella. Concentric rings cut from the umbrella are capable of maintaining a trapped wave for some time, but if the ring is too narrow, the wave cannot be started, or soon ceases after being started. Trapped waves can be started in two or three concentric rings cut from the umbrella and the wave revolves about the inner ring more often per second than about the outer ring, but the revolutions per second are not in exact inverse proportion to the mean diameters of the rings, or to the diameters of the inside tracks or holes in the rings. One subumbrella, 11.5 cm. in diam-

was cut into two rings and waves were trapped in them. The wave in the inner ring made 2.5 revolutions per second, and the wave in the outer ring made 2 revolutions per second.

The uncertainty as to the length of the pace-making circuit that the nerve impulse takes around the ring may be avoided by stretching the inner edge of the ring until it is of the same diameter as the outer, thus transforming the ring into a cylinder or tube.

Such a ring can be stretched further and behaves in a reversibly reversible manner. Since Mayer (1917) has shown that the rate of nerve conduction in *Cassiopea* depends on temperature and electric conductivity of the sea water, it should be noted

that all the experiments in this paper, unless otherwise stated, were done in sea water of 30° and Cl = 20. Mayer found a variation of only about 2.5 per cent over the range of pH = 8.26, and this variation includes experimental errors and changes due to unknown causes. In the present experiments the pH was about 8.2 unless otherwise stated. The only difficulty in estimating the rate of the contraction wave arose from

the fact that the rate is 1 to 5 per cent faster in the ring that has been stretched than in the ring that has just been relaxed, depending on the degree of recent stretching or relaxation.

If, however, the circumference of the ring is allowed to remain constant for 5 minutes after each short step of stretching or relaxation, the rate will approximate a mean value. This behavior of the ring may be regarded as a form of hysteresis, since the number of revolutions per second of the contraction wave

remains constant immediately after stretching or relaxation.

I do not see how this can be explained on the assumption

that the stretching of the neuraxon is the only factor, and it is significant to note that Carlson records no such hysteresis in the stretched nerve of the slug, where synapses are less numerous

and entirely absent. One explanation of the increased rate immediately after stretching might be the thinning of the plasma membrane of the neuraxon, the regeneration in thickness taking appreciable time.

Another suggestion is made that the immediate effect of stretching is increase in length of the neuraxon,

but that this may be proportionately less than the increase in length of the strip of tissue, since the nerve paths may be

straightened, and that the apparent hysteresis is due to the possibility that after the nerve is stretched synapses gradually

open, due to the tension, until the nerve paths are proportionately as zigzagged as at the start. This supposition may similarly be applied to the fact that by prodding a *Cassiopea* (having a trapped wave) with a stick, the number of revolutions of the wave per second is reduced, although no apparent increase in length of the conducting path remains. The local stretching of the subumbrella with the stick might break some of the synapses. Such complicated suppositions are not very useful, however, since they are not easily tested. A more probable hypothesis is given below in connection with the amplitude of the contraction wave. The significant fact is that (ignoring the period of readjustment) the ring may be stretched until the circumference is increased 72 per cent with practically no change in rate (mm. per second), although in order to accomplish this the number of revolutions per second or passages of the wave through the same tissue may be reduced 49 per cent. This is analogous to the effect of stretching a metallic wire on the passage of an electric current through it, with the difference that the process is completely reversible in *Cassiopea* after an increase of 72 per cent in length due to stretching. The results on two rings are as follows:

Length of circumference.	Rate (mm. per sec.)
mm.	
223	372
263	413
283	430
286	379
306	390
326	399
346	410
366	414
386	407
406	403
426	391
446	377
466	368
486	360
506	352
526	342

If the rate of wave propagation is the same, we would expect in umbrellas of *Cassiopeas* of different sizes that the number of revolutions of the wave per second would be inversely proportional to the diameter. The diameter is measured before the wave is started, and there is a progressive shrinkage in the diameter, due to starvation and decrease in volume, tension of the regenerating tissue after removal of the rhopalia, and increased tonus of the musculature (transforming the disc into a cup shape). This decrease in the diameter is associated with increase in revolutions per second, but agitation decreases the revolutions per second. It is therefore necessary to make the determinations under the same conditions for strictly comparative results. The following determinations were rough, but serve to indicate the general features.

Diameters. cm.	Revolutions of wave per sec.		
	Immediately.	After 24 hrs.	After prodding with a stick.
9.75	2.38	2.50	
10.50	1.79	2.17	2.04
10.50	1.75	2.17	2.04
10.75	1.75	2.13	
11.00	2.38	2.08	
11.50	1.72	2.22	
11.50	1.79	2.22	
12.50	1.67	1.72	1.66
12.80	1.67	2.00	
13.50	1.61	1.67	1.40
13.50	1.61	1.79	1.52
14.00	1.67	1.79	

A comparison of these experiments with those on the actual velocity of the wave shows the circumference of the potential pace-making circuit to be about 1.8 times the diameter. If the contraction wave is stopped by pressure and after a rest of some minutes or hours started again, it is slower than just before stopping, but if it is started again as quickly as possible after stoppage, the rate is the same. This effect of a rest seems to be associated with nutrition or recovery from fatigue, since the amplitude of the contraction wave is greater in the rested

umbrella, although the number of revolutions per second is decreased. Whether the actual rate of propagation is changed would be difficult to determine. It seems evident that the wave of nerve impulse precedes the wave of muscular contraction. The contraction of the muscle must stretch the adjacent regions, and hence stretch the region through which the nerve impulse is passing, thus increasing the distance traveled in one revolution and decreasing the revolutions per second. When the amplitude of contraction is increased, the stretching of the nerves is increased and the revolutions per second are decreased, but whether this can account for the total decrease has not been determined. The speeding up of the revolutions per second after the trapped wave is started is at first more abrupt and later more gradual, and is associated with both decrease in amplitude of contraction and decrease in diameter of the umbrella, due to starvation and contraction of scar tissue.

Evidently a change in the number of revolutions per second of the trapped wave or the amplitude of the contractions would cause an error in the determination of the effect of O_2 concentration on metabolism, and in order to estimate the limits of such errors, the relative metabolism of the muscle and other tissues was studied. The umbrella of three *Cassiopeas*, A, B, and C, of the same size (diameter = 11.5 cm.) were used (each for a series of experiments). In some experiments the rhopalia remained and the normal pulsations were generated, in others a trapped wave was induced, and in others the subumbrella was removed or merely the mesoglea left. The apparent (but slight) metabolism of the mesoglea was probably entirely due to a few remnants of epithelium and to bacteria, which always attack the mesoglea when the epithelium is removed. At any rate the metabolism of the mesoglea is too small to be of significance. The pH was 8.2 and the O_2 per liter 4.5 cc. at the beginning of each of the experiments.

The removal of the rhopalia in the umbrellas with trapped waves or without pulsations, reduced but slightly the amount of tissue. If we take the metabolism of the normal pulsating umbrella as 100, the exumbrella is about 14 and the resting subumbrella 60 with an addition of 26 for normal pulsations or 65 for trapped wave. Therefore, the neuromuscular tissue may per-

Individual.	O ₂ per hr.	Parts of umbrella used.
	cc.	
A	0.86	Umbrella with 0.64 pulsations per sec.
A	0.97	" " 0.64 " " "
A	0.158	Mesoglea + exumbrella only.
B	1.12	Umbrella with trapped wave, 1.47 per sec.
B	1.08	" " " " 1.47 " "
C	0.5	" not pulsating.
C	0.6	" " "
C	0.03	Mesoglea + bacteria.
D	0.88	Umbrella with 0.7 pulsations per second.
D	0.7	" not pulsating.

from about 26 to 46 per cent of the metabolism, and changes in rate or amplitude of the contraction wave are to be avoided as much as possible. In subsequent experiments, the manubrium and rhopalia were removed from the *Cassiopea*, and a C-shaped slit was made through the neuromuscular layer about one-third the radius from the outer margin of the umbrella, and a trapped wave induced by stimulation near the outer margin. The wave passed around the outer part of the subumbrella, and each time it passed the opening of the C it spread to the inner part, dividing into two equal waves, meeting on the far side with mutual destruction. In this way, the outer part, in which the trapped wave was first induced, was made pace-maker, and the result was a more permanent wave. The wave causes circulation of water against all parts of the epithelium except a small portion of the exumbrella which has a very low metabolism. If the oxygen concentration was reduced to zero, the wave stopped, and metabolism ceased until oxygen was readmitted. In one experiment an umbrella was kept 7 hours at zero O₂ concentration, then 3 hours with O₂ and a trapped wave, and 7 hours at zero O₂ concentration, during which it gave out no CO₂ or other acid products affecting the pH perceptibly. 30 seconds after it was taken out of the O₂-free chamber, a trapped wave was started and this constantly increased in amplitude for 10 minutes, at the end of which time the amplitude was normal. Oxygen was removed from sea water in various ways, with the air pump and agitation, by boiling, and by allowing a *Cassiopea* to remain

in it until the pulsations ceased, as seen through a peep hole in such a way that photosynthesis was practically avoided. In no case did the analysis show less than about 0.05 cc. per liter, but that amount probably entered with the KI and MnCl_2 solutions and around the ground stopper of the analysis bottle. At any rate, we should consider 0.05 cc. to be within the limit of error of the method if no correction be made for O_2 in the reagents.

The variation in metabolism after removal of the manubrium and initiation of a trapped wave is shown in the following table. The diameter of the umbrella was 11.5 cm. at the beginning of the first experiment and at the beginning of each experiment the pH was 8.2 and the O_2 per liter 4.5 cc.

Hrs. after operation.	O_2 used per hr.	Revolutions of wave per sec.
	cc.	
0	1.65	1.60
2	1.55	2.00
3	1.30	2.22
4	1.30	2.22
5	1.30	2.22
20	1.30	2.22

From the above table it is evident that the metabolic rate may vary rapidly for 3 hours after the manubrium is removed and the trapped wave is started, therefore in the subsequent experiments, the umbrella was not placed in the respiration chamber until these 3 hours had passed. The same umbrella used in the above experiments (but 21 hours after the operation) was used to determine the effect of reduced oxygen concentration. The average O_2 concentration during the experiment was 1.5 cc. per liter and the O_2 used per hour 1.03 cc., being a decline of 20 per cent in rate of oxidation with a reduction of the oxygen concentration to about half its original value. This and some later experiments are tabulated as follows:

At 3.3 cc. O ₂ per liter. O ₂ used per hr.	At 1.5 cc. O ₂ per liter. O ₂ used per hr.
1.3	1.03
1.3	1.00
1.4	1.10
1.48	1.15
1.44	1.25
1.35	1.05

These determinations show the oxidation is reduced about 20 per cent when the O₂ concentration is reduced about 50 per cent, or the oxidation increased about 25 per cent when the O₂ concentration is increased about 100 per cent.

DISCUSSION.

The above experiments show that the rate of oxidation varies with the concentration of oxygen in the sea water constantly circulated against the surface of the epithelium in which oxidation takes place. In the total absence of oxygen, no measurable quantity of CO₂ or other acid products is given out, and we may infer that the metabolism is suspended. If oxygen is readmitted after a suspension of the metabolism for 7 hours, the rate of metabolism rises apparently to the normal within 10 minutes. No anaerobic processes were detected during the absence of oxygen for 7 hours, but in the absence of oxygen for 16 hours, anaerobic or hydrolytic processes take place. No evidence was found to indicate that these anaerobic processes constituted the metabolism of the *Cassiopea*. On the contrary, a great multiplication of bacteria was associated with them, and the *Cassiopea* was partly dissolved and could not be revived by readmission of oxygen. We may assume that oxygen protects the *Cassiopea* from the attacks of bacteria (probably anaerobes). Since the bacteria enter from the surface, it seems probable that the *Cassiopea* might live indefinitely on so small a supply of oxygen that it is used in the superficial cells as fast as it diffuses into them and none reaches the deeper cells. If this be true, the variation in oxygen consumption with variation of supply might be the expression of a variation in the number of cells receiving oxygen. In other words, this would be a diffusion phenomenon.

Another hypothesis is that the concentration of oxygen at the seat of oxidation affects the rate. Warburg has shown that cell oxidation is associated with structure and that no enzyme solution has been obtained that will account for the vital oxidation of foodstuffs. If we call the structure or surface responsible for the oxidations the catalyst, it seems possible that the concentration of oxygen in the immediate vicinity of the catalyst influences the rate. This influence of concentration on rate might still be a diffusion phenomenon since the O_2 must diffuse toward the structure catalyst or oxidase molecule.

Owing to the excellent review of the literature on the subject of this paper by Krogh, it seems unnecessary to multiply references. Roughly speaking, and within physiological limits, animal oxidation is about doubled with 10° rise in temperature and this is shown here to be true of *Cassiopea*. In other words, oxidation is an exponential function of the temperature, as expressed in the following equation:

$$V_t = V_o \times 2^{\left(\frac{t}{10}\right)}$$

Where V_t is the velocity of oxidation at t° and V_o at 0° . This relation holds approximately true for a number of chemical reactions, and is supposed to be due partly to change in diffusion rate and partly to loosening of bonds in the reacting molecules, and to ionization. Diffusion depends on viscosity and molecular motion. A fall of temperature from 30° to 20° increases the viscosity of water about 25 per cent, and of a 40 per cent sucrose solution 42 per cent and of a 3 per cent gelatin solution 1,000 per cent (von Schroeder). The increase in viscosity of gelatin, however, does not cause a proportionate decrease in diffusion. We may assume that this fall of temperature may possibly cause about 30 per cent decrease in diffusion of O_2 within the cell, due to viscosity alone. Molecular motion is proportional to the absolute temperature, and hence is a minor factor in diffusion. Although diffusion accounts for only about a third of the change in rate of oxidation with temperature, it is undoubtedly a factor. Since diffusion affects oxidation and concentration gradient affects diffusion, it seems very probable that concentration of O_2 should affect oxidation, even though every cell received some

oxygen. Krogh interprets the experiments on warm-blooded animals as showing increased oxidation with increased oxygen tension, and reviews the work of Thunberg, showing the same effect to a greater degree on cold-blooded animals. Henze (1910, a) found that the oxidation rate of sea anemones and annelids varies with O_2 concentration (and of nudibranchs at low concentrations) and supposed that only a varying fraction of the cells received oxygen. His tables show that the oxidation rate of crustacea and jellyfish fell rapidly with time, but that in one series of determinations on *Pelagia*, the oxidation rate varied reversibly with O_2 concentration. In order to reduce the diffusion effect, he kept sea urchin eggs agitated in sea water and found that the oxidation rate apparently increased about 8 per cent on doubling the O_2 concentration. Henze attributes this apparent difference in respiration to faulty technique, but since the experiments have not been repeated with improved technique, we may assume that a real difference exists.

It is interesting to compare the metabolism of *Cassiopea* with that of jellyfish studied by Vernon. Since only the living cells metabolize, it would be an advantage to know the proportion of cellular tissue to the body weight, but there is no data on this subject. The skeletal structure, mesoglea, contains less organic matter than the cells, and hence the per cent of organic matter in the body is a partial indication of the cellular mass. The proportion of mesoglea increases with the size of the individual within the same species, but there can be no strict comparison between different species in this regard, and therefore the comparison is very crude. The number of cc. of O_2 per hour per kilo of body weight and per kilo of organic matter (= dry weight — weight of salts in equal volume of sea water) at 20° is given in the following table.

Species.	Weight of individual.	Cc. O_2 per hr.	
		Per kilo of body weight.	Per kilo of organic matter.
	gm.		
<i>Cassiopea zamachana</i>	100	12.5	1,040
<i>Carmarina hastata</i>	30	7.7	2,025
<i>Cestus veneris</i>	70	3.75	1,562

The agreement is about as close as could be expected.

Vernon has compared the metabolism (per unit weight of organic matter) of jellyfish, molluscs, tunicates, and vertebrates, and shown it to be remarkably constant. Krogh obtained greater differences when the total body weights were used (omitting jellyfish but including eggs and insects), but the differences probably do not exceed the differences in water content and in muscular activity. He found the metabolism of a young dog with body temperature reduced to 20° to be greater than that of cold-blooded animals at the same temperature, but if we reduce the metabolism of the average dog for 20°, using a reasonably high temperature coefficient, the agreement is more satisfactory. We should not expect close agreement unless water and mineral salts and fibrous tissue are excluded from the weight, and the activity of the nervous system is abolished. It seems probable, therefore, that the chief distinction in the calorimetry of warm-blooded and cold-blooded animals is in insulation. All warm-blooded animals are air breathing, and air brings much oxygen and takes away little heat. The center of a cluster of bees in winter may be 40° above that of the air bathing it.

The heat production in a 100 gm. *Cassiopea* at 30° is sufficient to raise its body temperature 0.14° per hour above that of the surrounding water, but no such difference in temperature has been observed because the heat generated is conducted away by the water bringing the oxygen. I found a fish weighing 1.4 gm. to use 0.825 cc. O₂ per hour at 30°, which is sufficient to raise its body temperature about 3° per hour, but during this time it was required to breathe 400 cc. of sea water, even though it removed half of the oxygen from water saturated with air at this temperature. The water circulating through the gills could remove the heat generated if the body temperature were 0.01° above that of the water. Since there was considerable conduction through the skin, and the fish probably removed much less than half the oxygen from the water in one passage through the gills, the body temperature was probably much less than 0.01° above that of the water. Rogers and Lewis could detect no difference between the body temperature of fish, salamanders, clams, and earthworms and the water in the thermostat in which they were placed, after they had been in the thermostat

long enough for equilibrium. They used a thermocouple, and one division of the galvanometer scale corresponded to 0.0042° .

It was shown that the metabolism of *Cassiopea* is proportional to its surface and not to its weight but to $W^{\frac{2}{3}}$. This is due to the fact that the metabolism is confined to the living cells and that these constitute a superficial epithelium, whose thickness is about the same in *Cassiopeas* of the range of sizes studied. We might use these results in an attempt to explain the so called surface law of warm-blooded animals. Dreyer, Ray, and Walker have shown that the blood volume and cross-sections of the aorta and trachea are proportional to $W^{\frac{2}{3}}$ (or surface). If animals are of the same shape (internally as well as externally) the cross-sections of all organs would be proportional to $W^{\frac{2}{3}}$ but the blood volume would be proportional to the weight (W). If the blood volume is proportional to $W^{\frac{2}{3}}$ the whole circulatory system would be nearly proportional to $W^{\frac{2}{3}}$ and owing to the close relation between the lungs and the blood, the volume of the lungs would be nearly proportional to $W^{\frac{2}{3}}$. The volume of the skin may be proportional to $W^{\frac{2}{3}}$ and the volume of the wall of the alimentary tract nearly so. The nerve, muscle, and glandular tissues are excitable, and hence their metabolism must vary. Variable components may be excluded from basal metabolism by definition but cannot all be excluded in making measurements. Only the skeleton can be said to have a metabolism proportional to W , and since the red bone marrow produces blood (erythrocytes) and this is proportional to the surface, the metabolism of some of the bones may be nearly proportional to the surface. Benedict has shown that great variations from the surface law exist, and hence it may be only accidental. The fact that the excitable tissues metabolize more per unit weight in small animals than in large (*i.e.*, proportional to a smaller power of W than unity) is true, not only for warm-blooded animals, but also for cold-blooded animals, to which the teleological principle of the surface law (in relation to heat regulation) does not apply. It seems possible, however, that the chief conditions necessary for the evolution of temperature-regulation in animals were: (1) air breathing, (2) large body size, (3) sensitivity to low temperatures, (4) variation of activity of excitable tissues with size, and

(5) epithelial type of architecture, (4) and (5) being common to cold-blooded animals.

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THE PREPARATION OF CYANAMIDE.

By A. E. OSTERBERG AND E. C. KENDALL.

(From the Chemical Laboratory of the Mayo Clinic, Rochester, Minn.)

(Received for publication, October 11, 1917.)

In the course of work undertaken in the chemical laboratory of the Mayo Clinic it became necessary to prepare considerable quantities of cyanamide in the free base form. The existing methods, particularly those having as a starting point thiourea¹ or calcium² cyanamide, were tried and subsequently abandoned because of the tedious procedures and poor yields obtained. Attempts were therefore made to modify the method, using calcium cyanamide as a starting point. The method of treating calcium cyanamide with aluminum sulfate³ was found to give low yields because of the slight solubility of calcium cyanamide necessitating many extractions of the starting material. In order to overcome this objection calcium cyanamide was treated with dilute sulfuric acid. This method, however, yielded variable results. Carbonic acid was then tried in place of sulfuric. It was found unnecessary to dissolve the calcium cyanamide in water before treatment. Simply allowing the carbon dioxide to bubble through an aqueous suspension of calcium cyanamide results in its almost quantitative decomposition. A method by which cyanamide may be easily obtained in a high state of purity, is described here in the hope that it may be of use to others.

The starting material was calcium cyanamide furnished by the Baker Chemical Company, having a total nitrogen content of 20 per cent. The yield was 55 gm. of pure cyanamide per 200 gm. of the calcium salt. This corresponds to a yield of 92 per cent of the theoretical.

¹ Volhard, J. *Jour. prakt. Chem.*, 1874, ix, 24.

² Baumann, E., *Ber. chem. Ges.*, 1873, vi, 1371.

³ Caro, N., *Z. angew. Chem.*, 1910, xxiii, 2405-17; abstr. in *J. Soc. Chem. Ind.*, 1911, xxx, 23.

Method.—200 gm. of calcium cyanamide were mixed in a 3 liter round-bottom flask with 1,500 cc. of distilled water. Into this mixture CO_2 was passed until a neutral or only slightly alkaline point was reached as determined by red litmus. The flask was kept immersed in cold water as the reaction causes a small rise in the temperature. We have observed that if the temperature is kept below 40° , there is very little loss of the ether-soluble product due to polymerization to dicyandiamide. The length of time necessary to precipitate the calcium varies with the amount of agitation. We have found it convenient to allow the precipitation to proceed over night, agitating but little.

The precipitate was filtered on a Buchner funnel, well washed with water, and the filtrate placed in a 3 liter round-bottom flask. A small amount of talcum was added to facilitate boiling and the solution was concentrated by distillation on a water bath *in vacuo*. The distillation⁴ was continued until the remaining solution when cooled under the cold water tap formed a solid crystalline mass, which was then extracted three times with absolute ether. The ether was distilled off on a water bath and the remaining solution concentrated over sulfuric acid *in vacuo*.

In this way 55 gm. of pure cyanamide were obtained in the form of deliquescent needles melting at 43° (when free from ether) and perfectly soluble in ether.

	Calculated for CNNH_2 :	Found:
N.....	66.67	67.00

⁴ However, if a higher temperature than 40°C. is required to drive off the last traces of water it is best to stop at this point and remove the remaining water *in vacuo* over sulfuric acid after the ether extraction. This may lead to contamination of the resulting product with dicyandiamide, if any is present, as dicyandiamide is soluble in a cyanamide solution in ether. However, after drying, a second ether extraction will remove all dicyandiamide.

**A STUDY OF AMINO NITROGEN AND GLUCOSE IN
LYMPH AND BLOOD BEFORE AND AFTER THE
INJECTION OF NUTRIENT SOLUTIONS
IN THE INTESTINE.**

BY BYRON M. HENDRIX AND JOSHUA E. SWEET.

*(From the Laboratory of Physiological Chemistry and the Laboratory of
Surgical Research, University of Pennsylvania, Philadelphia.)*

(Received for publication, October 4, 1917.)

INTRODUCTION.

The question of the absorption of protein by way of the lymph has not been considered since the true significance of protein digestion has been known. The earlier investigators were limited to the determination of total nitrogen as a means of following the products of protein digestion through the body. Newer methods make it possible and desirable to follow resorption of the products of protein digestion by the determination of amino nitrogen in the tissues and fluids of the body.

Asher and Barbera (1) fed egg albumin and by determining the apparent increase in total nitrogen in the lymph, thought they showed that a small amount of protein is absorbed through the lacteals. Mendel (2) repeated this experiment of Asher and Barbera, giving the animal Witte's peptone instead of egg albumin. His results showed a slight increase of total nitrogen in the lymph, but a smaller increase than that obtained by the former. He was inclined to believe that the lymph does not take up any protein from the intestines, as his slight increase in nitrogen seemed to be within the limits of experimental error. Howell (3) has demonstrated the presence of amino-acids in the lymph, but so far as we are aware, no quantitative determination of amino nitrogen in the lymph has been reported.

The presence of glucose in the lymph has been known for a long time, and as early as 1862 Gorup-Besanez (4) pointed out the fact that the percentage of sugar in the lymph is greater than in the blood. Ginsberg (5) thought the sugar in the blood is to be found in the plasma alone. He argued from this assumption that the lymph glucose did not show a real concentration over that of the blood but only an apparent one, since the removal of the red corpuscles, which make up from 30 to 40 per cent of the

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volume of the blood, would leave all of the sugar in the plasma, and, as he believed, in the lymph. However, Michaelis and Rona (6, 7) and von Hess and McGuigan (8) have shown that the amount of glucose in the red corpuscles is the same as in the plasma. Some other explanation must be sought for the relatively large amount of glucose in the lymph.

Von Mering (9) thought that the amount of glucose in the lymph is unaffected by a meal rich in sugar, but Ginsberg, working in Heidenhain's laboratory, showed that the amount of glucose in the lymph increases after sugar is injected into the intestine. He used dogs and rabbits and found the increase in both species of animals. Still he does not seem to have believed that sugar is resorbed directly by the lymph, but that it is first taken up by the blood and then secreted into the lymph spaces.

EXPERIMENTAL.

It has seemed worth while to make amino nitrogen determinations on blood and lymph collected before and after the injection, into the small intestine, of protein and amino-acid solutions and then compare the results with those obtained when sugar was injected and the glucose content of the blood and lymph determined. Dogs which had fasted about 18 hours were used in these experiments. They were anesthetized with ether, no morphine salts having been used.

Surgical Technique.—The ordinary thoracic fistula was used occasionally for the collection of lymph, but usually the collection was made through the left external jugular vein. This vein was tied off just below the entrance of the subclavian and also some distance above (at about the level of the upper edge of the thyroid gland); then all the veins (including the subclavian) flowing into the jugular between these ligatures were tied and cut. After this was done, an incision was made in the jugular, and in a few moments perfectly bloodless lymph flowed from a cannula inserted into the vein.

The lymph, thus obtained, came not only from the thoracic duct but also from the lymph vessels arising in the head and neck; however, our results did not show any difference between the samples of lymph collected in the different ways.

The operation, in preparing this type of lymphatic fistula, has the advantage of being more quickly performed by an experienced surgeon, and is one which a physiologist can do with little difficulty after a few attempts. The thoracic duct seems, at times, to end

in a sort of "delta," and under these conditions, the collection through the jugular vein is much more complete than it would be by the usual method.

Chemical Methods.—In preparing the blood and lymph for the amino nitrogen determination, the method of Van Slyke and Meyer (10) was followed. Usually 15 cc. of the blood or lymph were run into 85 cc. of 95 per cent alcohol and then 40 cc. of the alcohol added to make the volume up to 140 cc. The amino nitrogen was determined by the well known micro method of Van Slyke (11). The method of Lewis and Benedict (12) was followed in the glucose determinations.

Experimental Results.—The results of four experiments are given in the following tables. In two of these experiments, solutions of Fairchild Brothers and Foster's "peptone" were used; in one of the others, milk was used; and in the fourth, a solution of amino-acids was injected. These injections were all made into the intestine at about the level of the lower end of the pancreas. It is to be noted that the quantity of protein used was relatively large in each case, probably somewhat in excess of the amount present in the intestine at any time under normal conditions.

Experiment 1. March 21, 1917.

Operation complete, 10.15 a.m.

Sample No.	Material.	Time of collection.	Amino N per 100 cc.
			mg.
1	Blood.	10.30	3.6
2	Lymph.	10.15-10.30	1.9

Injected 500 cc. of 10 per cent "peptone" solution at 10.40.

3	Lymph.	10.30-10.45	4.0
4	"	10.45-11.00	6.5
5	Blood.	11.00	10.2
6	Lymph.	11.00-11.14	13.8
7	"	11.14-11.28	19.0
8	Blood.	11.28*	4.7

* Animal dead.

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Experiment 2. April 12, 1917.

Operation complete, 10.20 a.m.

Sample No.	Material.	Time of collection.	Amino N per 100 cc.
			mg.
1	Lymph.	11.00-11.18	Lost.
2	Blood.	11.13	3.4
3	Lymph.	11.08-11.20	3.1

Injected 500 cc. of milk at 11.29.

4	Lymph.	11.20-11.31	2.9
5	"	11.31-11.44	3.6
6	"	11.44-11.51	3.2 (P)
7	"	11.51-11.58	Lost.
8	"	11.58-12.06	4.9
9	Blood.	12.09	4.4
10	Lymph.	12.06-12.13	6.1
11	"	12.13-12.22	7.2
12	"	12.22-12.35	8.3
13	"	12.35	6.6
14	"		4.5
15	"	12.55-1.02	4.4
16	Blood.	1.02	3.2

Experiment 3. April 23, 1917.

Operation complete, 11.15 a.m.

Sample No.	Material.	Time of collection.	Amino N per 100 cc.
			mg.
1	Lymph.	11.15-11.55	3.7
2	Blood.	11.55	5.3

Injected solution of amino-acids containing 4.2 gm. amino nitrogen so lution
at 12.00.

3	Lymph.	11.55-12.20	6.0
4	"	12.20-12.40	9.2
5	Blood.	12.45	6.5
6	Lymph.	12.40-1.00	9.7
7	Blood.	1.20	6.5
8	"	1.20	8.6 (P)

Experiment 4. May 16, 1917.

Operation complete, 11.15 a.m.

Sample No.	Material.	Time of collection.	Amino N per 100 cc.
1	Lymph.	11.15-11.40	4.7
2	Blood.	11.45	6.3

Injected 500 cc. of 5 per cent "peptone" solution at 12.30.

3	Lymph.	12.30-1.05	8.2
4	"	1.07-1.28	6.0 (?)
5	Blood.	1.10	6.4
6	Lymph.		8.7
7	Blood.	1.42	4.9
8	Lymph.		6.4
9	Blood.		6.8
10	Lymph.		7.4

It will be observed from the tables given above that, in the fasting dog, the amount of amino nitrogen in the lymph was less than in the blood. Sometimes the difference was quite marked, while at other times, it was insignificant. This is the reverse of the relation of the amount of glucose in the blood and lymph. After the protein or amino-acid solution is placed in the intestine, the amino nitrogen content of the blood and lymph is increased. Although this increase was not always quite regular, the increase in the blood was only once or twice more than 50 per cent, while in the lymph, the increase was usually more than 100 per cent and in many cases much greater. In Experiment 1, the amino nitrogen in the lymph rose to a trifle over 19 mg. per 100 cc., which is practically ten times what it was in the control period. It is to be pointed out that, after the injection of protein or amino-acid solution into the intestine, not only the increase in, but also, the absolute amount of amino nitrogen in the lymph, becomes greater than in the blood.

It is to be noted that the blood was collected from the general circulation, sometimes from the jugular vein, and at other times from the femoral artery. Since the liver is believed to take up a portion of the amino acids from the portal blood, possibly,

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if the blood had been collected from the portal or one of the mesenteric veins, there would have been little or no difference in the amino nitrogen content of the blood and lymph.

These experiments would seem to show that the amino-acids in the intestine may be taken up by both blood and lymph. They also suggest that the amount taken up by each of these fluids is dependent upon the volume of the two fluids which are flowing through the walls, and especially, the villi of the intestine during the period of resorption. This would mean, of course, that only a relatively small amount of protein nitrogen is taken up by the lymph, since the amount flowing from the intestine is certainly small as compared with the flow of blood.

We have already mentioned the work of Ginsberg in which he showed that the amount of glucose in the lymph increases after the injection of sugar into the intestine. The following tables from three of our experiments show that we have been able to confirm these results. These experiments were carried out in exactly the same way as those in which we were studying the resorption of amino-acids, except that glucose solutions were introduced into the intestine and 2 cc. samples of blood and lymph were collected for glucose determinations.

Experiment 5. May 29, 1917.

Operation complete, 11.45 a.m.

Sample No.	Material.	Time of collection.	Per cent of glucose.
1	Lymph.	12.25	0.225
2	"	12.30	0.225
3	Blood.	12.35	0.145
4	"	12.35	0.145

Injected 50 gm. glucose dissolved in 250 cc. of water at 1.05.

5	Lymph.	1.15	0.250
6	Blood.	1.15	0.136
7	Lymph.	1.30	0.321
8	Blood.	1.35	0.176
9	Lymph.	2.00	0.321
10	Blood.	2.05	0.180
11	Lymph.	2.30	0.310

Experiment 6. June 16, 1917.

Operation complete, 10.45 a.m.

Sample No.	Material.	Time of collection.	Per cent of glucose.
1	Lymph.	10.50	0.300
2	Blood.	10.55	0.176

Injected 50 gm. glucose dissolved in 250 cc. of water at 11.00.

3	Lymph.	11.20	0.306
4	Blood.	11.20	0.228
5	Lymph.	11.45	0.360
6	Blood.	11.55	0.247
7	Lymph.	12.10	0.434
8	Blood.	12.10	0.271

Experiment 7. August 10, 1917.*

Operation complete, 11.10 a.m.

Sample No.	Material.	Time of collection.	Per cent of glucose.
1	Lymph.	11.15	0.173
2	Blood.	11.19	0.125

Injected 50 gm. glucose dissolved in 250 cc. of water at 11.20.

3	Lymph.	11.35	0.164
4	Blood.	12.20	0.281
5	Lymph.	12.35	0.300
6	Blood.	12.50	0.300
7	Lymph.	1.03	0.281

* In this experiment, blood was collected from the mesenteric veins.

Special attention should be called to Experiment 7. It was carried out just as the other glucose experiments except that the blood samples *were collected from the mesenteric veins*. This experiment indicates, if it does not clearly show, that the glucose content of the thoracic lymph and the portal blood is the same. This does not account for the amount of glucose in the lymph of the fasting dog. None of these experiments throw any light on that fact.

It would seem, on purely physical grounds, that the concentration of glucose and of amino-acids in the lymph and blood of

the villi of the intestine would be identical, as the processes of diffusion and osmosis would operate to eliminate any inequalities, unless a semipermeable membrane is present to prevent the passage of these substances in one direction or the other. As it is generally agreed that these substances pass into the blood through the walls of the capillaries, there does not appear to be any physical basis for believing that they would pass into the capillaries to such an extent that the blood would be richer in these compounds than the fluid which is found in the intracellular spaces surrounding the blood vessels. Heidenhain (13) thought the structure of the villi of the intestine practically prevents the entrance of sugar and other crystalloids into the lymph, as he, as well as Schäfer (14) and others, have shown that the blood vessels lie adjacent to the basement membrane of the epithelial cells which cover the villi, while the lacteal is centrally located. He did not take account of the fact that the intracellular spaces are really continuous with the lymphatic system so that, in reality, the blood system can be regarded as no nearer to the resorbing surface than the lymphatics.

Therefore, it is suggested that the practically complete resorption of sugar and amino-acids by the blood is to be accounted for by the almost infinitely large volume of blood flow as compared with that of the lymph.

SUMMARY.

1. Less amino nitrogen is found in the thoracic lymph than in the blood of a fasting dog.
2. After the injection of milk, "peptone," or amino-acid solutions into the intestine, the amino nitrogen in both the systemic blood and lymph increases, but the amount in the lymph is greater than in the blood.
3. Ginsberg's findings, that the introduction of sugar solutions into the intestine increases the amount of glucose in the lymph, are confirmed. The old observation, that the amount of glucose in the lymph is greater than in the blood, has also been confirmed.
4. The amount of sugar in the blood of the mesenteric veins and the lymph after the introduction of sugar into the intestine, seems to be practically the same.

5. It is suggested that the practically complete absorption of protein and carbohydrate by the blood is not due to a selective resorption, but to the almost infinitely large volume of blood, as compared to the volume of lymph, which flows through the walls of the intestine.

We wish to express our thanks to Dr. A. E. Taylor for suggesting this problem to us, also to Mrs. Mary S. Witherspoon for technical assistance in carrying out some of our experiments.

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The precipitate was filtered on a Buchner funnel, well washed with water, and the filtrate placed in a 3 liter round-bottom flask. A small amount of talcum was added to facilitate boiling and the solution was concentrated by distillation on a water bath *in vacuo*. The distillation⁴ was continued until the remaining solution when cooled under the cold water tap formed a solid crystalline mass, which was then extracted three times with absolute ether. The ether was distilled off on a water bath and the remaining solution concentrated over sulfuric acid *in vacuo*.

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STUDY OF AMINO NITROGEN AND GLUCOSE IN LYMPH AND BLOOD BEFORE AND AFTER THE INJECTION OF NUTRIENT SOLUTIONS IN THE INTESTINE.

By BYRON M. HENDRIX AND JOSHUA E. SWEET.

From the Laboratory of Physiological Chemistry and the Laboratory of Surgical Research, University of Pennsylvania, Philadelphia.)

(Received for publication, October 4, 1917.)

INTRODUCTION.

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Von Mering (9) thought that the amount of glucose in the lymph is unaffected by a meal rich in sugar, but Ginsberg, working in Heidenhain's laboratory, showed that the amount of glucose in the lymph increases after sugar is injected into the intestine. He used dogs and rabbits and found the increase in both species of animals. Still he does not seem to have believed that sugar is resorbed directly by the lymph, but that it is first taken up by the blood and then secreted into the lymph spaces.

EXPERIMENTAL.

It has seemed worth while to make amino nitrogen determinations on blood and lymph collected before and after the injection, into the small intestine, of protein and amino-acid solutions and then compare the results with those obtained when sugar was injected and the glucose content of the blood and lymph determined. Dogs which had fasted about 18 hours were used in these experiments. They were anesthetized with ether, no morphine salts having been used.

Surgical Technique.—The ordinary thoracic fistula was used occasionally for the collection of lymph, but usually the collection was made through the left external jugular vein. This vein was tied off just below the entrance of the subclavian and also some distance above (at about the level of the upper edge of the thyroid gland); then all the veins (including the subclavian) flowing into the jugular between these ligatures were tied and cut. After this was done, an incision was made in the jugular, and in a few moments perfectly bloodless lymph flowed from a cannula inserted into the vein.

The lymph, thus obtained, came not only from the thoracic duct but also from the lymph vessels arising in the head and neck; however, our results did not show any difference between the samples of lymph collected in the different ways.

The operation, in preparing this type of lymphatic fistula, has the advantage of being more quickly performed by an experienced surgeon, and is one which a physiologist can do with little difficulty after a few attempts. The thoracic duct seems, at times, to end

in a sort of "delta," and under these conditions, the collection through the jugular vein is much more complete than it would be by the usual method.

Chemical Methods.—In preparing the blood and lymph for the amino nitrogen determination, the method of Van Slyke and Meyer (10) was followed. Usually 15 cc. of the blood or lymph were run into 85 cc. of 95 per cent alcohol and then 40 cc. of the alcohol added to make the volume up to 140 cc. The amino nitrogen was determined by the well known micro method of Van Slyke (11). The method of Lewis and Benedict (12) was followed in the glucose determinations.

Experimental Results.—The results of four experiments are given in the following tables. In two of these experiments, solutions of Fairchild Brothers and Foster's "peptone" were used; in one of the others, milk was used; and in the fourth, a solution of amino-acids was injected. These injections were all made into the intestine at about the level of the lower end of the pancreas. It is to be noted that the quantity of protein used was relatively large in each case, probably somewhat in excess of the amount present in the intestine at any time under normal conditions.

Experiment 1. March 21, 1917.

Operation complete, 10.15 a.m.

Sample No.	Material.	Time of collection.	Amino N per 100 cc.
			mg.
1	Blood.	10.30	3.6
2	Lymph.	10.15-10.30	1.9

Injected 500 cc. of 10 per cent "peptone" solution at 10.40.

3	Lymph.	10.30-10.45	4.0
4	"	10.45-11.00	6.5
5	Blood.	11.00	10.2
6	Lymph.	11.00-11.14	13.8
7	"	11.14-11.28	19.0
8	Blood.	11.28*	4.7

* Animal dead.

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Experiment 2. April 12, 1917.

Operation complete, 10.20 a.m.

Sample No.	Material.	Time of collection.	Amino N per 100 cc.
			mg.
1	Lymph.	11.00-11.18	Lost.
2	Blood.	11.13	3.4
3	Lymph.	11.08-11.20	3.1

Injected 500 cc. of milk at 11.29.

4	Lymph.	11.20-11.31	2.9
5	"	11.31-11.44	3.6
6	"	11.44-11.51	3.2 (?)
7	"	11.51-11.58	Lost.
8	"	11.58-12.06	4.9
9	Blood.	12.09	4.4
10	Lymph.	12.06-12.13	6.1
11	"	12.13-12.22	7.2
12	"	12.22-12.35	8.3
13	"	12.35	6.6
14	"		4.5
15	"	12.55-1.02	4.4
16	Blood.	1.02	3.2

Experiment 3. April 23, 1917.

Operation complete, 11.15 a.m.

Sample No.	Material.	Time of collection.	Amino N per 100 cc.
			mg.
1	Lymph.	11.15-11.55	3.7
2	Blood.	11.55	5.3

Injected solution of amino-acids containing 4.2 gm. amino nitrogen solution at 12.00.

3	Lymph.	11.55-12.20	6.0
4	"	12.20-12.40	9.2
5	Blood.	12.45	6.5
6	Lymph.	12.40-1.00	9.7
7	Blood.	1.20	6.5
8	"	1.20	8.6 (?)

Experiment 4. May 16, 1917.

ation complete, 11.15 a.m.

Sample No.	Material.	Time of collection.	Amino N per 100 cc.
			mg.
1	Lymph.	11.15-11.40	4.7
2	Blood.	11.45	6.3

Injected 500 cc. of 5 per cent "peptone" solution at 12.30.

3	Lymph.	12.30-1.05	8.2
4	"	1.07-1.28	6.0 (?)
5	Blood.	1.10	6.4
6	Lymph.		8.7
7	Blood.	1.42	4.9
8	Lymph.		6.4
9	Blood.		6.8
10	Lymph.		7.4

will be observed from the tables given above that, in the dog, the amount of amino nitrogen in the lymph was less than in the blood. Sometimes the difference was quite marked, but at other times, it was insignificant. This is the reverse of the relation of the amount of glucose in the blood and lymph. When the protein or amino-acid solution is placed in the intestine, the amino nitrogen content of the blood and lymph is increased. Though this increase was not always quite regular, the increase in the blood was only once or twice more than 50 per cent, while in the lymph, the increase was usually more than 100 per cent, and in many cases much greater. In Experiment 1, the amino nitrogen in the lymph rose to a trifle over 19 mg. per 100 cc., which is practically ten times what it was in the control period. It is to be pointed out that, after the injection of protein or amino-acid solution into the intestine, not only the increase in, but also, the absolute amount of amino nitrogen in the lymph, becomes greater than in the blood.

It is to be noted that the blood was collected from the general circulation, sometimes from the jugular vein, and at other times from the femoral artery. Since the liver is believed to take up a large portion of the amino acids from the portal blood, possibly,

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if the blood had been collected from the portal or one of the ~~mes-~~enteric veins, there would have been little or no difference in ~~the~~ amino nitrogen content of the blood and lymph.

These experiments would seem to show that the amino-acids in the intestine may be taken up by both blood and lymph. They also suggest that the amount taken up by each of these fluids is dependent upon the volume of the two fluids which are flowing through the walls, and especially, the villi of the intestine during the period of resorption. This would mean, of course, that only a relatively small amount of protein nitrogen is taken up by the lymph, since the amount flowing from the intestine is certainly small as compared with the flow of blood.

We have already mentioned the work of Ginsberg in which he showed that the amount of glucose in the lymph increases after the injection of sugar into the intestine. The following tables from three of our experiments show that we have been able to confirm these results. These experiments were carried out in exactly the same way as those in which we were studying the resorption of amino-acids, except that glucose solutions were introduced into the intestine and 2 cc. samples of blood and lymph were collected for glucose determinations.

Experiment 5. May 29, 1917.

Operation complete, 11.45 a.m.

Sample No.	Material.	Time of collection.	Per cent of glucose.
1	Lymph.	12. 25	0. 225
2	"	12. 30	0. 225
3	Blood.	12. 35	0. 145
4	"	12. 35	0. 145

Injected 50 gm. glucose dissolved in 250 cc. of water at 1.05.

5	Lymph.	1. 15	0. 250
6	Blood.	1. 15	0. 136
7	Lymph.	1. 30	0. 321
8	Blood.	1. 35	0. 176
9	Lymph.	2. 00	0. 321
10	Blood.	2. 05	0. 180
11	Lymph.	2. 30	0. 310

Experiment 6. June 15, 1917.

tion complete, 10.45 a.m.

Sample No.	Material.	Time of collection.	Per cent of glucose.
1	Lymph.	10.50	0.300
2	Blood.	10.55	0.176

Injected 50 gm. glucose dissolved in 250 cc. of water at 11.00.

3	Lymph.	11.20	0.306
4	Blood.	11.20	0.228
5	Lymph.	11.45	0.360
6	Blood.	11.55	0.247
7	Lymph.	12.10	0.434
8	Blood.	12.10	0.271

Experiment 7. August 10, 1917.*

tion complete, 11.10 a.m.

Sample No.	Material.	Time of collection.	Per cent of glucose.
1	Lymph.	11.15	0.173
2	Blood.	11.19	0.125

Injected 50 gm. glucose dissolved in 250 cc. of water at 11.20.

3	Lymph.	11.35	0.164
4	Blood.	12.20	0.281
5	Lymph.	12.35	0.300
6	Blood.	12.50	0.300
7	Lymph.	1.03	0.281

In this experiment, blood was collected from the mesenteric veins.

Special attention should be called to Experiment 7. It was carried out just as the other glucose experiments except that the blood samples were collected from the mesenteric veins. This experiment indicates, if it does not clearly show, that the glucose concentration of the thoracic lymph and the portal blood is the same. This does not account for the amount of glucose in the lymph of the dog. None of these experiments throw any light on that

It would seem, on purely physical grounds, that the concentration of glucose and of amino-acids in the lymph and blood of

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the villi of the intestine would be identical, as the processes of diffusion and osmosis would operate to eliminate any inequalities, unless a semipermeable membrane is present to prevent the passage of these substances in one direction or the other. As it is generally agreed that these substances pass into the blood through the walls of the capillaries, there does not appear to be any physical basis for believing that they would pass into the capillaries to such an extent that the blood would be richer in these compounds than the fluid which is found in the intracellular spaces surrounding the blood vessels. Heidenhain (13) thought the structure of the villi of the intestine practically prevents the entrance of sugar and other crystalloids into the lymph, as he, as well as Schäfer (14) and others, have shown that the blood vessels lie adjacent to the basement membrane of the epithelial cells which cover the villi, while the lacteal is centrally located. He did not take account of the fact that the intracellular spaces are really continuous with the lymphatic system so that, in reality, the blood system can be regarded as no nearer to the resorbing surface than the lymphatics.

Therefore, it is suggested that the practically complete resorption of sugar and amino-acids by the blood is to be accounted for by the almost infinitely large volume of blood flow as compared with that of the lymph.

SUMMARY.

1. Less amino nitrogen is found in the thoracic lymph than in the blood of a fasting dog.
2. After the injection of milk, "peptone," or amino-acid solutions into the intestine, the amino nitrogen in both the system, blood and lymph increases, but the amount in the lymph is greater than in the blood.
3. Ginsberg's findings, that the introduction of sugar solution into the intestine increases the amount of glucose in the lymph, are confirmed. The old observation, that the amount of glucose in the lymph is greater than in the blood, has also been confirmed.
4. The amount of sugar in the blood of the mesenteric vein and the lymph after the introduction of sugar into the intestine seems to be practically the same.

5. It is suggested that the practically complete absorption of protein and carbohydrate by the blood is not due to a selective resorption, but to the almost infinitely large volume of blood, as compared to the volume of lymph, which flows through the walls of the intestine.

We wish to express our thanks to Dr. A. E. Taylor for suggesting this problem to us, also to Mrs. Mary S. Witherspoon for technical assistance in carrying out some of our experiments.

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NUTRITIVE FACTORS IN ANIMAL TISSUES. I.*

BY THOMAS B. OSBORNE AND LAFAYETTE B. MENDEL.

WITH THE COOPERATION OF EDNA L. FERRY AND ALFRED J. WAKEMAN.

(From the Laboratory of the Connecticut Agricultural Experiment Station
and the Sheffield Laboratory of Physiological Chemistry in
Yale University, New Haven.)

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Our early experience¹ in attempts to feed white rats with artificial mixtures of purified foodstuffs led us to the conviction that something in addition to the proteins, fats, carbohydrates, and inorganic salts, which were then regarded as the only essentials of an adequate diet, was necessary for the normal nutrition of these animals. This was made evident by the rapid growth of young rats fed with mixtures containing a part of the carbohydrate and all of the inorganic salts in the form of our so called "protein-free" milk, whereas those fed with chemically similar diets which lacked the small amount of the unknown constituents of milk grew little if at all. These observations coincided with earlier ones by Stepp² (on mice) and those of Hopkins³ who had independently reached the same conclusion. Subsequent investigations by ourselves and others have greatly extended the knowledge of the need of both growing and adult animals for those still chemically unknown substances, which are now generally designated as food hormones, or vitamins.⁴ Of these, two types

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

¹ Osborne, T. B., and Mendel, L. B., *Carnegie Institution of Washington, Publication No. 158*, pts. i and ii, 1911.

² Stepp, W., *Biochem. Z.*, 1909, xxii, 452; *Z. Biol.*, 1911-12, lvii, 135.

³ Hopkins, F. G., *J. Physiol.*, 1912, xlv, 425.

⁴ For a statement of the development of certain of our ideas on these topics see Osborne and Mendel, *Biochem. J.*, 1916, x, 534.

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appear to exist: one soluble in fats, the other soluble in water. They have been shown to be present in the following animal and vegetable products.

Food Hormones.

Fat-soluble.	Water-soluble.
Butter fat. ⁹	Milk. ^{9, 5}
Egg yolk fat. ⁷	Rice. ¹²
Cod liver oil. ⁸	Wheat embryo. ^{13, 27}
Beef fat. ⁹	Cotton seed. ¹⁴
"Oleo-oil" margarines. ^{9, 25}	Pancreas. ¹⁵
Pig kidney fat. ^{10, 11}	Maize kernel. ¹⁶
Maize kernel. ^{11, 18, 20}	Wheat " " ¹⁷
Rye. ¹¹	Oat " " ¹⁸
Wheat embryo. ^{11, 13}	Kidney beans. ¹⁹
" kernel. ¹¹	Yeast. ²⁴
Cotton seed. ^{14, 20, 27}	Soy beans. ²⁶
Cabbage leaves. ^{18, 23}	Maize embryo. ²⁸
Clover " " ^{18, 23}	Peanut meal. ²⁸
Oat kernel. ¹⁸	
Soy beans. ^{20, 26}	
Sunflower seed. ²¹	
Alfalfa leaves. ^{22, 23}	
Flax seeds. ^{22, 23}	
Hemp seed. ²²	
Millet seeds. ^{22, 23}	

⁵ Osborne and Mendel, *Carnegie Institution of Washington, Publication No. 156*, pt. ii, 1911; *Science*, 1911, xxxiv, 722; *J. Biol. Chem.*, 1912, xii, 473; 1912-13, xiii, 233.

⁶ Osborne and Mendel, *J. Biol. Chem.*, 1913, xv, 311; 1913-14, xvi, 423; 1914, xvii, 401; 1915, xx, 379. McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1913, xv, 167.

⁷ Osborne and Mendel, *J. Biol. Chem.*, 1914, xvii, 401; 1915, xx, 379. McCollum and Davis, *J. Biol. Chem.*, 1913, xv, 167. MacArthur, C. G., and Luckett, C. L., *J. Biol. Chem.*, 1915, xx, 161.

⁸ Osborne and Mendel, *J. Biol. Chem.*, 1914, xvii, 401. McCollum and Davis, *J. Biol. Chem.*, 1915, xx, 641.

⁹ Osborne and Mendel, *J. Biol. Chem.*, 1915, xx, 379.

¹⁰ McCollum and Davis, *J. Biol. Chem.*, 1915, xx, 641.

¹¹ McCollum and Davis, *J. Biol. Chem.*, 1915, xxi, 179.

¹² McCollum and Davis, *J. Biol. Chem.*, 1915, xxiii, 181.

¹³ McCollum, E. V., Simmonds, N., and Pitz, W., *J. Biol. Chem.*, 1916, xxv, 105.

¹⁴ Richardson, A. E., and Green, H. S., *J. Biol. Chem.*, 1916, xxv, 307; 1917, xxxi, 379.

It is to be noted that in the above lists milk and pancreas are the only products of animal origin that have as yet been tested for the presence of the water-soluble vitamine. Indirect evidence that animal tissues may contain this hormone is afforded by studies of the protective, curative, or antineuritic properties of some of these in relation to experimental polyneuritis or beriberi. That the substance which induces the remarkable recoveries which have been described in these cases is identical with the water-soluble hormone which is so essential for growth and maintenance is as yet merely a matter of conjecture.

Suzuki, Shimamura, and Odake²⁹ reported that an alcoholic extract of horse meat was unable to prevent the death of mice fed on a diet of polished rice; and that dogs fed exclusively on polished rice and boiled horse meat became greatly emaciated and ill in 3 to 4 weeks. Cooper,³⁰ who has reviewed the scanty but often suggestive earlier literature up to 1914, has compared the antineuritic properties of animal tissues of various sorts with respect to their relative potency in preventing or curing avian polyneuritis induced by feeding polished rice. The following excerpts from his protocols will serve to indicate the general results.

- ¹⁵ Eddy, W. H., *J. Biol. Chem.*, 1916, xxvii, 113.
- ¹⁶ McCollum, Simmonds, and Pitz, *J. Biol. Chem.*, 1916-17, xxviii, 153.
- ¹⁷ McCollum, Simmonds, and Pitz, *J. Biol. Chem.*, 1916-17, xxviii, 211.
- ¹⁸ McCollum, Simmonds, and Pitz, *J. Biol. Chem.*, 1917, xxix, 341.
- ¹⁹ McCollum, Simmonds, and Pitz, *J. Biol. Chem.*, 1917, xxix, 521.
- ²⁰ McCollum, Simmonds, and Pitz, *Am. J. Physiol.*, 1916, xli, 361.
- ²¹ McCollum, Simmonds, and Pitz, *Proc. Soc. Exp. Biol. and Med.*, 1916, xiii, 129.
- ²² McCollum, Simmonds, and Pitz, *J. Biol. Chem.*, 1917, xxx, 13.
- ²³ McCollum, E. V., *J. Am. Med. Assn.*, 1917, lxviii, 1379.
- ²⁴ Osborne and Mendel, *J. Biol. Chem.*, 1917, xxxi, 149.
- ²⁵ Halliburton, W. D., and Drummond, J. C., *J. Physiol.*, 1917, li, 235.
- ²⁶ Osborne and Mendel, *J. Biol. Chem.*, 1917 xxxii, 369. Daniels, A. L., and Nichols, N. B., *J. Biol. Chem.*, 1917, xxxii, 91.
- ²⁷ Osborne and Mendel, *J. Biol. Chem.*, 1917, xxix, 289.
- ²⁸ Osborne and Mendel, unpublished data.
- ²⁹ Suzuki, U., Shimamura, T., and Odake, S., *Biochem. Z.*, 1912, xliii, 89.
- ³⁰ Cooper, E. A., *J. Hyg.*, 1912, xii, 436; 1914, xiv, 12.

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Amount of Animal Products Necessary to Prevent Polyneuritis. According to Cooper.

	In terms of natural foodstuff.	In terms of dry weight.
	gm.	gm.
Ox voluntary muscle.....	20	5.0
" cardiac "	5	1.7
" cerebrum.....	6	1.2
" cerebellum.....	12	2.4
" liver.....	3	0.9
Cow's milk.....	>35	>3.5
Sheep cerebrum.....	8 to 15	1.6 to 3
Fish voluntary muscle.....	>10	>2
Egg yolk.	3	1.5
Cheese.....	>8	>5.6

In 1913 Funk³¹ made the following statement: In the animal kingdom unheated milk and egg yolk are rich in vitamins; these foods must supply the great need of vitamins on the part of young, growing organisms; furthermore these substances are found in meat, especially abundantly in heart muscle, in brain, and probably in most animal tissues. In a tabular summary he refers to meat juice, meat extract, and slightly roasted meat as containing vitamins; and to sterilized meat extract and soup meat as being devoid of vitamins. We assume that this refers to the antineuritic properties of the substances mentioned. It is unnecessary to offer a critique of the significance of the results of these earlier statements from the standpoint of the vitamin hypothesis of the present time, which takes into account all of the possible factors which may be involved in an inadequate diet.^{24, 32} The problems of growth were not considered.

In view of the great importance of food hormones in the diet and the wide use of animal tissues of various kinds for food it has seemed desirable to undertake a comprehensive study of this question. Furthermore, it has seemed possible that in parts of

³¹ Funk, C., *Munch. med. Woch.*, 1913, 2614. "In der Tierwelt ist rohe Milch, das Eigelb reich an Vitaminen; diese Stoffe müssen den grossen Bedarf an Vitaminen bei jungen wachsenden Organismen decken; ferner befinden sich diese Substanzen im Fleisch, besonders reichlich im Herzmuskel, im Gehirn und wahrscheinlich in den meisten Tiergeweben."

³² McCollum, E. V., and Pitz, W., *J. Biol. Chem.*, 1917, **xxi**, 229.

the various animals which are now little or not at all used for human consumption there may be some which are particularly rich in the water-soluble food hormone and that consequently these, if not suitable for human food, could at least be more advantageously fed to growing domestic animals than is now the case.

The results of our experiments with beef muscle and pig liver which are described in this paper were of such a character that we are now actively engaged in extending this study to a greater variety of animal products. We have thus far investigated beef muscle, meat extract, the tissue residue from this meat extract, and dried pig liver, following the plan of our earlier studies with rats. The substance under investigation was fed as the sole source of protein and water-soluble vitamins in the diet, combined with suitable carbohydrates, fats, and inorganic salts, or merely as the sole source of water-soluble food hormone or vitamins in an otherwise adequate food mixture.

The "*meat powder*" was prepared by grinding fresh lean round of beef very fine, drying on pans in a current of hot air, then in an oven at 105°C., and grinding to a powder. The "*meat extract*" was prepared by grinding finely chopped lean round of beef through a Nixtamal mill with nearly three times its weight of distilled water. The thin pulp was then boiled for a few minutes over a free flame, filtered, and the extract concentrated over a steam bath or in a current of hot air, nearly dried in a vacuum desiccator and then in an oven at 105°C.²³ In this way 17.3 per cent of meat extract in terms of the total meat solids—each calculated on a water-free basis—was obtained. This is a yield considerably larger than that usually reported for the water-soluble non-coagulable components of meat; it is doubtless due to the extreme degree of comminution obtained by grinding with much water in a suitable mill. Before mixing with the other ingredients in the food the meat extract was dissolved in a little water, mixed with a quantity of cornstarch and dried on pans in a current of hot air. The "*meat residue*" was prepared by pressing the solid portion remaining from the filtration of the extract, in a hydraulic press, reextracting it with water, drying in a current of hot air, and finally in an oven at 105°C. The *dried liver* was prepared by removing the larger blood vessels from the fresh liver as completely as possible, drying the material in a current of hot air, and finally in an oven at 105°C.

²³ This method of drying is essentially the same as we have employed in making "protein-free milk." Our extensive experience with this material has given us no reason to believe that the vitamins are damaged to any great extent by the heating.

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The results of the nutrition experiments are shown in graphic form in the accompanying charts. Both the meat powder and the meat extract seem to be deficient in the water-soluble food hormone. When yeast, "protein-free milk," corn germ, or wheat embryo—all tested sources of the water-soluble food hormone—were added to the diet, a rapid response in growth was made by the rats; whereas without them nutritive failure invariably ensued. From this it is evident that both the meat powder and the meat residue are suitable as sources of *protein* in the diet when the other essential ingredients are present in sufficient amounts. This needs emphasis in view of the fact that meat residues such as remain after the preparation of soups from muscle tissue are often discarded as inferior food products. The proteins which they still contain are entirely adequate for nutrition in growth.

The meat extract apparently contains at least a small amount of the water-soluble food hormone; for the rats on the edestin-meat extract food have grown somewhat better than the majority of our animals which have been entirely deprived of this vitamine. The quantity of meat extract in this food, however, is equivalent to almost twice as much as that in the meat which the rats on the meat powder foods received.

In striking contrast to these results obtained with the muscle tissue and its extract are those furnished by the experiments with the dried pig liver. They demonstrate that this organ contains both adequate protein and a large proportion of water-soluble vitamine. These results parallel the findings of Cooper in respect to the relative antineuritic properties of muscle and liver, and also agree with those of Eddy,¹⁸ which "seemed to prove conclusively that the water-soluble portion of the alcoholic extract of pancreas contains a substance that is capable of inducing marked increase in growth." It is to be noted that both liver and pancreas, in contrast to muscle tissue, are exceptionally rich in glandular cells.

CHARTS.

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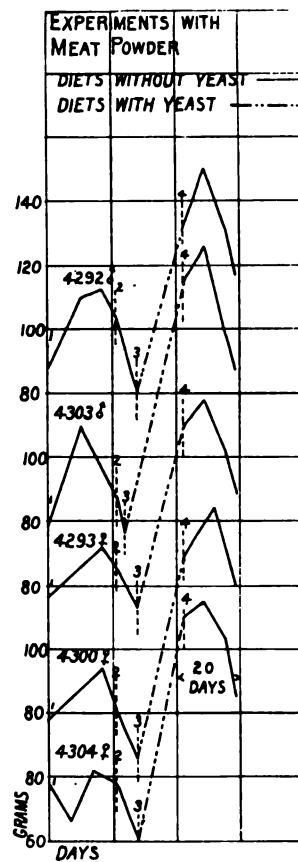


CHART I.

CHART I. Showing the failure of young rats to grow on a diet of *meat powder*, starch, fats, and a salt mixture (Periods 1, 2, and 4) except when a small amount of yeast was furnished in addition (Period 3). The addition of butter fat in Period 2 in place of lard supplied the fat-soluble food hormone without averting the nutritive decline. The quantity of yeast added in Period 3, during which the animals grew very rapidly, amounted to only 0.2 gm. per day. This was fed separately. The withdrawal of yeast in Period 4 was soon followed by nutritive disaster although this occurred somewhat less speedily than in the experiments with *meat residue*, Chart II, Period 3, doubtless owing to the small quantity of water-soluble food hormone present in the whole *meat powder* but entirely lacking in the *meat residue*. The highly successful growth when all of the other essentials were present (Period 3) shows that the meat proteins *per se* are entirely adequate for nutrition in growth.

The composition of the foods in the different periods was as follows:

	Period 1.	Periods 2 and 4.	Period 3.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Meat powder.....	20	20	20
Salt mixture.....	4	4	4
Starch.....	61	61	61
Lard.....	15		
Butter fat.....		15	15
Dried yeast.....			0.2 gm. <i>per diem</i> .
Nitrogen content.....	2.4	2.4	2.6

The salt mixture used in all of these experiments had the following composition:

	<i>gm.</i>		<i>gm.</i>
CaCO ₃	134.8	Citric acid + H ₂ O.....	111.1
MgCO ₃	24.2	Fe citrate 1½ H ₂ O.....	6.34
Na ₂ CO ₃	34.2	KI.....	0.020
K ₂ CO ₃	141.3	MnSO ₄	0.079
H ₂ PO ₄	103.2	NaF.....	0.248
HCl.....	53.4	K ₂ Al ₂ (SO ₄) ₂	0.0245
H ₂ SO ₄	9.2		

The chemicals used were analyzed and allowance was made for moisture, etc. The acids were mixed and the carbonates and ferric citrate added to them. The traces of KI, MnSO₄, NaF, and K₂Al₂(SO₄)₂ were added as solutions of known concentrations. The final resulting mixture was evaporated to dryness in a current of air at 90-100°C., and ground to a fine powder.

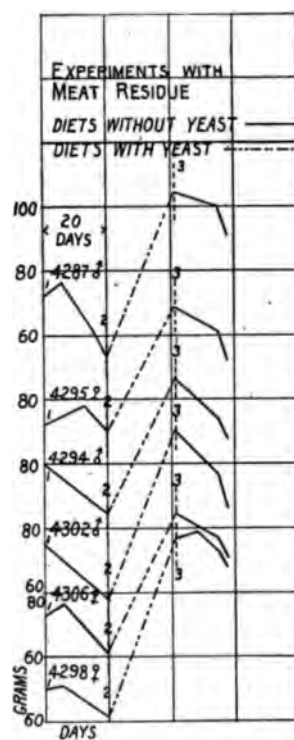


CHART II.

CHART II. Showing experiments comparable with those in Chart I except that meat residue from which the extractives had been removed was used in place of the meat powder. Here, too, there was a failure of nutrition except when dried yeast (amounting to 2 per cent of the food mixture and equal to about 0.2 gm. per day) was present in the ration (Period 2). As explained under Chart I, the failure on the foods without added sources of water-soluble food hormone are more prompt in these meat residue experiments than in the trials with the meat powder. The proteins of the meat residue are thus proved to be entirely adequate for growth when all other essentials are furnished in the ration.

The composition of the food mixtures is as follows:

	Period 1.	Period 2.	Period 3.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Meat residue.....	18.5	18.5	18.5
Salt mixture*.....	4.0	4.0	4.0
Starch.....	53.5	51.5	53.5
Butter fat.....		18.0	18.0
Lard.....	24.0	6.0	6.0
Dried yeast.....		2.0	
Nitrogen content.....	2.8	3.0	2.8

* For composition of salt mixture see legend for Chart I.

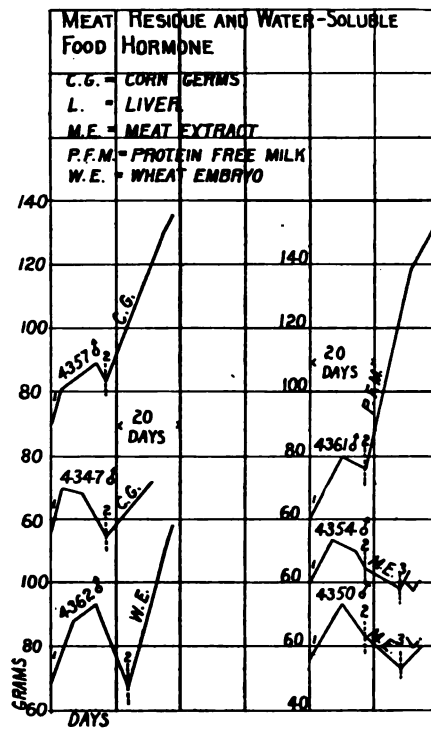


CHART III.

CHART III. Showing the nutritive decline owing to the lack of water-soluble food hormone in the meat residue (Period 1) and the prompt response in growth when various sources of the water-soluble food hormone such as protein-free milk, corn germs, wheat embryo, and liver were added in subsequent periods. The presence of 3 per cent of meat extract (equivalent to the amount originally present in the meat) in the diet of Rats 4362, 4357, 4347 (Period 1) evidently did not furnish sufficient of the hormone to avert the decline. Even 5 per cent of meat extract in the diet was insufficient when tested with Rats 4350 and 4354 (Period 2). The results afford striking examples of the importance of properly combining ordinary foodstuffs so that sufficient quantities of all of the dietary essentials are furnished.

The composition of the food mixtures was as follows:

	Period 1.		Period 2.				Period 3.
	Rats	Rats	Rats	Rat	Rats	Rat	Rats
	4347 4357 4362	4350 4354 4361	4347 4357	4362	4350 4354	4361	4350 4354
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Meat residue.....	14	18.5	14	14	18.5	18.5	18.5
Meat extract.....	3				5.0		
Corn germs.....			5				
Wheat embryo.....				5			
Dried liver.....							5.0
Protein-free milk.....						28.0	
Salt mixture.....	4	4.0	4	4	4.0		4.0
Starch.....	55	53.5	53	53	48.5	29.5	48.5
Butter fat.....	18	18.0	18	18	18.0	18.0	18.0
Lard.....	6	6.0	6	6	6.0	6.0	6.0
Nitrogen content.....	2.4	2.8	2.2	2.3	3.3	3.0	3.3

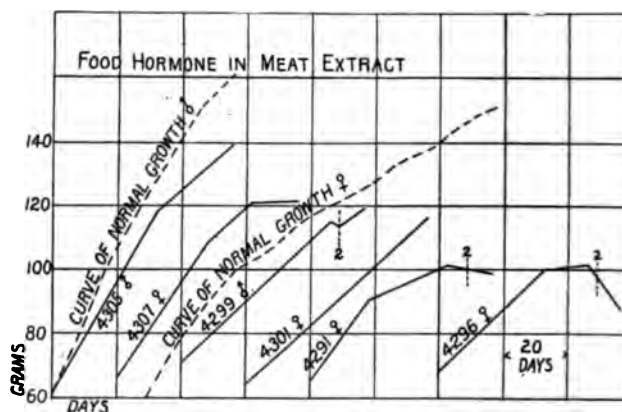


CHART IV. Experiments in which 5 per cent of meat extract was used as the sole source of food hormones. The growth of these rats was decidedly better than experience with vitamine-free diets of comparable composition would lead us to expect; but the normal rate of growth has not been sustained. The failure at this stage was not due to the lack of the fat-soluble hormone; for growth was not improved by the addition of butter fat in Period 2. Furthermore inasmuch as rats recorded in Chart III failed to grow even with an abundance of butter fat in the diet, when meat extract served as the source of the water-soluble hormone, but responded promptly to other sources of the latter, the failure of the rats on this chart cannot be attributed to a lack of fat-soluble hormone. We, therefore, conclude that the meat extract contains at best only a small proportion of the water-soluble food hormone.

The composition of the food mixtures was as follows:

	Period 1.	Period 2.
	<i>per cent</i>	<i>per cent</i>
Edestin.....	18	18
Salt mixture.....	4	4
Meat extract.....	5	5
Starch.....	50	50
Lard.....	23	5
Butter fat.....		18
Nitrogen content.....	3.65	3.65

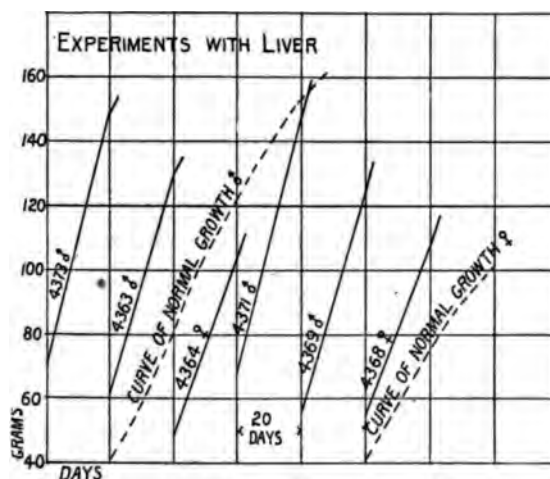


CHART V. Showing the high efficiency of dried pig liver as the source of both protein and water-soluble food hormone for nutrition during growth. The contrast between the success of the experiments with liver and those of muscle products shown in the preceding charts is most striking.

The composition of the food mixtures was as follows:

	Rats 4373 4363 4364	Rats 4371 4369 4368
	per cent	per cent
Dried liver.....	23	23
Salt mixture*.....	4	4
Starch.....	50	50
Butter fat.....	18	
Lard.....	5	23
Nitrogen content.....	2.4	2.4

* For composition of salt mixture see legend for Chart I.

APPLICATIONS OF GAS ANALYSIS.

II. THE CO₂ TENSION OF THE VENOUS BLOOD AND THE CIRCULATION RATE.

By YANDELL HENDERSON AND A. L. PRINCE.

(From the Physiological Laboratory, Yale Medical School, New Haven.)

(Received for publication, October 10, 1917.)

The determination of the CO₂ tension of the arterial blood by analysis of the air from a deep expiration—the CO₂ tension of the so called “alveolar air” of Haldane and Priestley, or as we shall call it the “arterial pulmonary air”—has come to have great importance both experimental and clinical. It is probable that a simple method of determining the CO₂ tension, and the oxygen also when wanted, in the venous blood, or “venous pulmonary air,” will be almost equally useful.

The importance of such a method lies in the fact that it affords a basis for measuring the volume of the blood stream, the so called circulation rate. At present there is not and there is little prospect for the development of a method along any other lines for the measurement of this supremely important function.

But, as has been pointed out by Christiansen, Douglas, and Haldane (1), once we know how to determine the gas tensions of the blood coming to the right heart and lungs, we have all the factors necessary to determine the minute-volume of the blood stream. The essentials of such a method are as follows: (1) Saturate a sample of the subject's blood with a gas mixture of the venous tensions (venous pulmonary air) and determine by analysis the CO₂ content of this blood. This gives the volumes per cent of CO₂ of the blood in the right heart. (2) Treat another sample of the subject's blood with arterial pulmonary air (obtained by a sudden deep expiration) and analyze. This gives the CO₂ content of the blood in the left heart. (3) Have the subject breathe through double valves into a graduated spirometer for a measured time. Read the volume expired and analyze a sample of the

air for CO_2 . The product of the figures thus obtained gives the amount of CO_2 expired per minute.

From these three measurements we can derive the circulation rate. For suppose that the individual expires 10 liters of air a minute containing 4 per cent CO_2 . Suppose the arterial blood is found to contain 52 volumes per cent of CO_2 and the venous 60. Then the heart is pumping 5 liters of blood a minute, *i.e.*, $(10 \times 0.04) \div (0.60 - 0.52) = 5$. This volume divided by the pulse rate is the systolic discharge.

Obviously, even without this blood analysis, venous pulmonary air high in CO_2 is a strong indication of a circulation slow in proportion to the subject's metabolism,—in other words, an inefficient heart.

For the determination of the venous CO_2 tension a method has been described by Christiansen, Douglas, and Haldane which consists in making up in a bag or spirometer a gas mixture of about the CO_2 tension expected. After a preliminary deep expiration the subject inhales the contents of the bag, holds it in his lungs a few seconds, or breathes it back and forth from and into the bag, and then exhales deeply into the bag. If on analysis the CO_2 percentage in the air in the bag is increased above what it was before it was breathed, it is evident that the mixture was originally too low in CO_2 . If it is decreased, then it was too high. A new mixture is accordingly made and tried until one is attained which may be inhaled and exhaled, during a period less than the time it takes blood to pass from the left around to the right heart, without undergoing any appreciable change in gas composition. This mixture is of course equivalent (provided certain requirements regarding oxygen are complied with) to the gas tensions of the venous blood.

In considering this method the first thought is naturally: Why not merely have the subject hold his breath until the air in the lungs is changed to the venous composition by the flow of blood through the pulmonary circulation? In fact, however, the pulmonary air volume is so large and the attainment of the venous tension takes so many seconds that before it is complete some of the blood makes the round of the circulation and returns to the lungs abnormally rich in CO_2 . Accordingly the rise of CO_2 in the lungs does not stop at the venous tension, but as

Christiansen, Douglas, and Haldane find, continues to rise. Some observations of our own (Fig. 1) suggest that the level at which the curve becomes nearly horizontal is that of the CO_2 tension of the tissues at rest (about 7.8 per cent).

The incapacity of this method to give the venous pulmonary air has been confirmed in an extensive paper by Wardlaw (2), who has also studied the rise of CO_2 in a bag of air which the subject rebreathes. Before seeing Wardlaw's paper we had found that at least in some subjects a series of determinations of this sort (Higgins-Plesch method) with increasing numbers of breaths

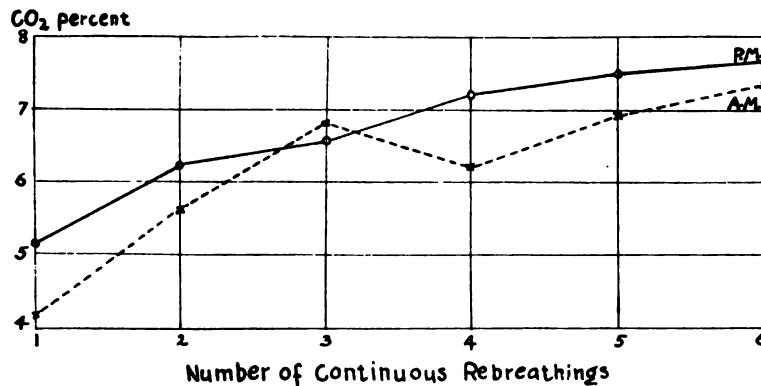


FIG. 1. Two series of CO_2 determinations on the air in a bag after the number of deep rebreathings (inspiration 5 seconds, expiration 5 seconds) indicated below the curves. Note that a step occurs in both curves at about 6.6 per cent CO_2 but earlier in p.m. (after dinner) than in a.m. without breakfast and in low CO_2 production.

into and from a bag (allowing time to recover normality between observations) yields a curve of CO_2 rise in which a short but distinct step or level part occurs (at about the third breath with the subject at rest and even earlier after a meal or light exercise), which probably indicates the venous CO_2 tension. Such curves are shown in Fig. 1.

The method which we shall here describe differs from the above in that the time of holding the breath or rebreathing is reduced to less than the period required for any of the blood to complete a circulation, but the rebreathing is repeated at intervals of several minutes using the same air over and over again. It suggested

itself from the fact that the Higgins-Plesch method of determining the alveolar air often gives figures which are considerably too high for the arterial CO_2 tension and approximate the venous tension. In practice the method is considerably simpler than the following description, requiring only a single analysis of the contents of the bag of air after it has been taken into the lungs six or seven times with adequate intervals for the return of normal conditions. On first trial of the method it is well, however, to follow through the full technique which is as follows:

The subject starts with the lungs well filled and makes the deepest possible expiration into an empty rubber bag. The bag is closed and a sample of its contents analyzed. A CO_2 percentage of from 4.5 to 5.5 is usually found. After a few minutes of normal breathing the subject empties his lungs as deeply as possible (not into the bag) and then quickly inhales the entire content of the bag. He holds this air in his lungs for about 5, 10, or 15 seconds (according as the subject is at rest or has been exercising, see below) and then exhales as deeply as possible into the bag. Another analysis is made and a higher CO_2 percentage is found, and the procedure is repeated again and again (with intervals to allow for return of normal conditions of respiration and circulation) until constancy of composition in the air is found in successive analyses. When the subject is at rest this occurs usually after about the fifth inhalation of the air. (A variation of this procedure is, instead of holding the lungs full, to inspire for 5 seconds and expire 5 seconds one or more times.)

Proof that the tension of CO_2 in this air is at least approximately that of the venous blood is afforded by the following procedure. A bag of air containing about 9 per cent of CO_2 is prepared and is inhaled and analyzed successively. The CO_2 instead of rising falls gradually in a curve which becomes level at the same percentage as that reached when ordinary air is started with. A double experiment of this sort is shown in Fig. 2. Evidently in this subject (A. L. P.) the venous CO_2 tension at rest is about 6.8.

A similar determination of the venous CO_2 tension during work is shown in Fig. 3.

Consideration of these observations leaves one question still to be answered; *viz.*, in any individual and under various rates

of blood flow and CO_2 production, how many seconds should the air of the bag be held in the lungs or rebreathed? To estimate the probably correct answer we may suppose (1) that after the preliminary deep expiration (not into the bag) the lungs contain 2,000 cc. of residual air at 5.5 per cent CO_2 , and that the air inspired at the fifth rebreathing from the bag is 3,000 cc. of 6.5

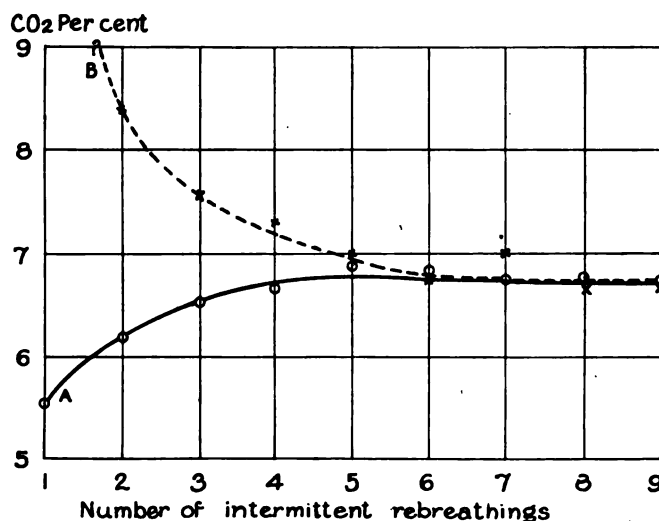


FIG. 2. In this diagram the numbers on the abscissa represent successive rebreathings at intervals sufficient for return of normal breathing.

Curve A. Beginning with a full expiration into the bag, each observation thereafter represents the CO_2 percentage obtained by rebreathing the contents of the bag *only once* (inspiration 5 seconds, expiration 5 seconds).

Curve B. Beginning with inspiration of an artificially prepared 10 per cent CO_2 mixture in the bag the succeeding observations were made as in the case of Curve A. It will be noted that after the sixth intermittent breath, the CO_2 in the bag is in equilibrium with the venous blood CO_2 tension.

per cent CO_2 . Then the lungs will contain 5,000 cc. of air of 6.1 per cent CO_2 . Suppose the CO_2 production of the body is 300 cc. per minute or 5 cc. per second. Then the 5,000 cc. of air held in the lungs would be raised from 6.1 to 6.6 per cent CO_2 in a period of more than 5 seconds, but probably of less than 10 seconds and would then have reached an equilibrium with the

CO₂ tension of the venous blood coming to the lungs, if the latter were 6.6 per cent. Repetition would yield the same figure.

Evidently if the air were held so long that some of the blood made the circuit from the lungs through the tissues and back to the lungs the CO₂ content of the air would again rise, and a false high equilibrium would be obtained. If the air is held in the lungs too short a period a false low result would be reached.

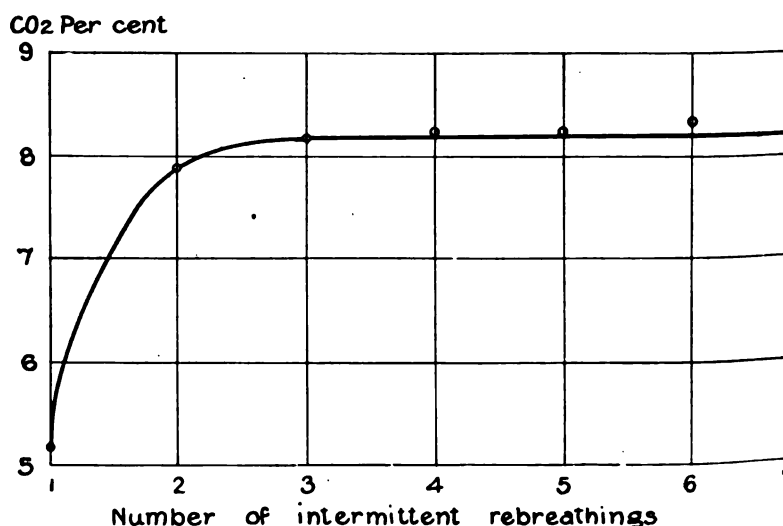


FIG. 3. Observations conducted as in Curve A, of Fig. 2. In this experiment the subject was performing work at the rate of 167 kilogrammeters per minute. Note the increased CO₂ tension, as compared with the resting conditions of Fig. 2, and the more rapid attainment of equilibrium of air and blood.

To test these considerations a series of separate experiments was made in some of which the air was held in the lungs for only 3 seconds, in others 5, 10, 15, and 20 seconds, respectively, at each inspiration from the bag. The results are summarized in Fig. 4 and indicate that under conditions of rest a period of 20 seconds is too long and one of 3 seconds is too short. Between 6 and 16 seconds the results are identical and therefore correct. So for a normal man at rest 10 seconds may be adopted as a safe and correct period. During even moderate exercise 5 seconds appears a sufficiently long period to hold the air in the lungs repeatedly.

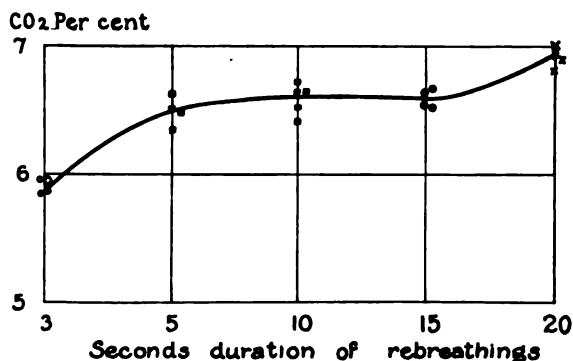


FIG. 4. Each dot represents the mean of several determinations; *i. e.*, a complete experiment (see Fig. 2, Curve A) after gaseous equilibrium had been established, made after the sixth to the twelfth intermittent rebreathings under conditions of rest. The time indicated represents the duration of the intermittent breaths (inspiration plus expiration). Note that the true (or at any rate the same) CO₂ equilibrium is established if the period is any length between 6 and 16 seconds. Outside of these limits false equilibria are established. It is evident that this time factor will need to be shortened during exercise.

SUMMARY.

A simple method of determining the CO₂ tension of the venous blood is described and some of its applications to measurement of the circulation and to the efficiency of the heart are indicated. For all the analyses required in this method the apparatus described in the first paper of this series is suitable (3).

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APPLICATIONS OF GAS ANALYSIS.

III. AN APPARATUS FOR THE STUDY OF THE RESPIRATORY EXCHANGE IN SMALL ANIMALS.

By A. L. PRINCE.

(*From the Physiological Laboratory, Yale Medical School, New Haven.*)

(Received for publication, October 10, 1917.)

The method here described has been found useful in the study of various aspects of metabolism in small animals, and also in the teaching of students. An experiment, including the analyses and calculations may easily be completed within an hour. By duplicating the apparatus, it is possible to conduct two or more experiments at once. In most of our work white rats have been used. Usually the animal is asleep and apparently entirely inactive throughout the experiment. Even under these conditions, and even with an unvarying respiratory quotient, we find that in small animals marked variations in the respiratory exchange occur due doubtless to movements too slight for observation.

Method.

The apparatus is shown in Fig. 1. At the beginning of the experiment, bottles A and B, having a capacity of 12 and 2.5 liters respectively, are filled with water from a faucet shown in the diagram. A film of mineral oil, a few cc. deep, is floated above the water in bottle A. We have found by experiment that this film prevents the loss of CO₂ from the sample collected in the bottle. Owing to the high solubility of CO₂ in water, grave errors in the results occur without this protective film. (If, as Bayliss claims, oil dissolves more gas than does water, the stagnant character of the oil robs its solvent power of any practical importance in this connection.)

The animal is placed in chamber C, which can be hermetically sealed on a glass plate by means of vaseline. This chamber

holds a thermometer giving the temperature of the chamber at any time during the experiment. After the animal is placed in the chamber, the clip on the siphon from bottle B is opened and as the water falls, the room air is drawn in through a Müller valve (D). The air passes from the valve into the chamber by a small coiled lead pipe, closed at its end and perforated with pin-point holes distributed throughout its length. This permits a thorough diffusion of the air passing through the chamber. The outlet from bottles A and B is regulated to deliver about 700 cc. per minute. The capacity of bottle B, therefore, permits a

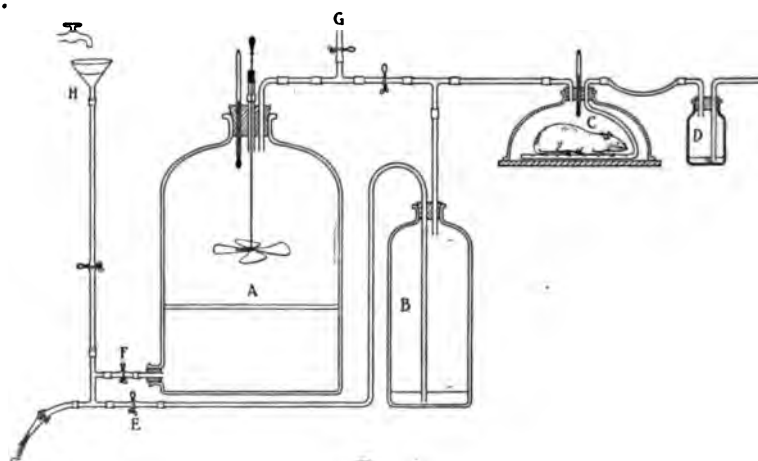


FIG. 1.

preliminary period of several minutes for the attainment of uniform conditions before the actual experiment begins. In order to get as nearly as possible a uniform flow throughout the experiment the tube through which the water escapes from the bottles is carried down 100 cm. below the surface of the water in the bottles (this is not shown in the figure), and as the surface falls the outlet of the tube is gradually lowered so as to keep this head constant, particularly at the beginning and end of the experiment.

At the end of this preliminary period the clip attached to siphon E is closed and the clip F is opened. The water is allowed to flow out of bottle A for a measured period of time, generally 15 minutes. The amount of air which has passed through the

chamber during this time is determined at once by the level of water in bottle A, which is calibrated in cubic centimeters. After the sample of air has been collected, all connections which might allow entrance of outside air to the bottle are shut off and the

TABLE.

Date.	Time.	Weight.	Barometer.	Corrected vol- ume of air.	Duration of ex- periment.	Analysis.		CO ₂ per min.	O ₂ per min.	R. Q.	Temperature of gas sample.
						CO ₂	O ₂				
Rat on diet of bread and water.											
1917		gm.	mm.	cc.	min.	per cent	per cent	cc.	cc.		°C.
Mar. 28	3.00	333	760	6343.4	14	1.457 1.452	19.348	6.60	7.34	0.90	21
Apr. 2	3.00	330	761	6442.1	14	1.455	19.39	6.55	7.22	0.90	17.7
" 3	3.00	331	765	6446.3	14	1.76	19.10	7.95	8.65	0.918	19.0
" 9	3.00	335	761	6419	14	1.337	19.52	5.99	6.55	0.90	18.5
Mean.....								6.77	7.44	0.90	
Animal without food 6 hours before first experiment.											
Apr. 12	3.05	330	760	7301	21.5	2.025 2.022	18.47 18.46	6.77	8.8	0.77	19.5
" 12	4.09	330	760	7301	21.5	2.050 2.027	18.38 18.47	6.8	8.9	0.76	19.5
" 12	4.34	330	760	7318	21.5	2.230 2.180	18.18 18.28	7.4	9.7	0.76	19.0
Mean.....								6.99	9.1	0.763	

animal is removed from the chamber. The air in bottle A is thoroughly mixed by the fan shown in the diagram and its temperature taken. By allowing water to enter bottle A by way of funnel H a sample of any desired size is withdrawn, either into a rubber bag or directly into a gas analyzer from the T connection G. This air is then analyzed for oxygen and CO₂.¹

¹ Paper IV of this series.

Knowing the total quantity and the temperature of the air collected in unit time, the barometric pressure and the percentage of CO_2 and oxygen in the air, we have all the elements needed to calculate the CO_2 elimination and oxygen consumption per minute and the respiratory quotient, and also (by the indirect method) the calories expended.²

The type of results obtained is shown in the accompanying table. It will be noted that the weight of the animal did not change materially during the period of these experiments over 2 weeks, and that the respiratory quotient remained practically constant at 0.9 when the animal was on a diet of bread and water, and at 0.76 when fasting. In view of the uniformity of the respiratory quotient under fixed conditions, and in view of the fact that the animal was asleep and apparently quiescent throughout all of the experiments, the variations in the respiratory exchange for the short periods observed are much greater than would be expected, amounting to 12 per cent.

It is well known that the rate of metabolism varies directly with the surface weight ratio. This evidently applies strictly only in cases where there is a marked difference in the surface area of the animals under study. In a series of experiments comprising more than 50 animals weighing from 114 to 213 gm. the differences in metabolic rates observed did not follow this law consistently. We have found that within these weight limits, the CO_2 elimination per gm. per minute varied from 0.03 cc. CO_2 to 0.033 cc.; in the case of the oxygen consumption per gm. per minute from 0.033 to 0.038 cc.

² Haldane, J. S., *Methods of Air Analysis*, London, 1912.

RELATIONSHIP BETWEEN CHOLESTEROL AND CHOLESTEROL ESTERS IN THE BLOOD DURING FAT ABSORPTION.*†

By ARTHUR KNUDSON.

(From the Laboratory of Biological Chemistry, Medical Department of Union University, Albany Medical College, Albany.)

(Received for publication October 11, 1917.)

Besides the true fats (compounds of glycerol and fatty acids) other substances closely related to fats are utilized in the animal economy. Some of these, including cholesterol, are essential constituents of every living cell. Cholesterol exists in the body tissues in both a free condition and a combined form as esters of higher fatty acids; such as palmitic, oleic, and stearic.

Cholesterol in the free condition has been known to act as a neutralizing substance to a number of different poisons (antihemolytic action against saponin,¹ tetanolysin,² etc.). Very little, however, is known of the use of esters of cholesterol or the part that cholesterol plays in the metabolism of fatty acids. It has been shown that during absorption of free cholesterol from the intestine it is partially esterified and appears so in the chyle and *vice versa* during absorption of cholesterol esters they are partially hydrolyzed.³ During absorption of fat free from cholesterol Bloor⁴ found that the cholesterol of the blood remains constant and this is confirmed in the experiments reported here, although some investigators^{5,6} have claimed an increase of cholesterol. In recent investigations,^{7,8} on the partition

* A report of this work was presented before the American Chemical Society, in Boston, September, 1917.

† This work was supported in part by a grant from the Elizabeth Thompson Science Fund.

¹ Ransom, F., *Deutsch. med. Woch.*, 1901, xxvii, 194.

² Abderhalden, E., and Le Count, E. R., *Z. exp. Path. u. Therap.*, 1905-06, ii, 199.

³ Mueller, J. H., *J. Biol. Chem.*, 1915, xxii, 1.

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⁸ Bloor, J., *J. Biol. Chem.*, 1916, xxvi, 417.

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of lipoids in normal and pathological blood cholesterol was found to bear a constant relation to the other blood lipoids. The fact that cholesterol maintains this constant relation to fatty acids and lecithin even in severe lipemia would seem to indicate that it plays an important part in fat metabolism.

In most animals a definite balance is found to exist between free and bound cholesterol in normal blood.⁹⁻¹¹ In normal human blood there was also found a constant relation between free and bound cholesterol, which was also maintained in most pathological conditions. It was likewise found to support the claim that there are few if any cholesterol esters in the corpuscles.¹² This constant relation between free and bound cholesterol would tend to give further support to the assumption that cholesterol takes an active part in fat metabolism. Therefore, it was deemed desirable to get a better understanding of the relation of these two forms so as to obtain further solution of the problem of their physiology.

In the present work determinations of the balance between cholesterol and cholesterol esters in the blood during the period of fat absorption were made. Along with these determinations of cholesterol and its esters the other lipid constituents of the blood (total fatty acids and lecithin) were determined in order to confirm the results reported by Bloor.¹³

Dogs were given a feeding of fat only and blood samples were taken at once and at 2 hour intervals for 8 hours. Previous to the fat feeding the dogs were fasted for 24 hours in order to be sure of the blood being free from ingested fat. The blood samples were drawn from the jugular vein into a hypodermic syringe (containing a little powdered oxalate) and run at once into a centrifuge tube containing a little oxalate. 3 cc. of the whole blood were then extracted and made up to 100 cc. volume according to Bloor's procedure.¹³ Then the tube was centrifuged for 10 minutes at 2,000 revolutions per minute and relative volume of corpuscles and plasma noted. 3 cc. of the plasma were then extracted as was the whole blood.

Cholesterol, cholesterol esters, total fat, and lecithin were determined on each specimen of whole blood and the plasma.

⁹ Fraser, M. T., and Gardner, J. A., *Proc. Roy. Soc., B*, 1909-10, lxxii, 559.

¹⁰ Klein, W., and Dinkin, L., *Z. physiol. Chem.*, 1914, xcii, 302.

¹¹ Mueller, J. *Biol. Chem.*, 1916, xxv, 561.

¹² Bloor, W. R., and Knudson, A., *J. Biol. Chem.*, 1916, xxvii, 107.

¹³ Bloor, J. *Biol. Chem.*, 1916, xxiv, 227.

Cholesterol was determined by Bloor's¹³ method using 10 cc. of the blood extracts for each determination. In the determination of cholesterol the method as reported recently¹⁴ was used and from 20 to 25 cc. of the blood extracts were required for each determination. Total fat (total fatty acids plus cholesterol) was determined by Bloor's method.¹⁵ The value for total fatty acids used in the tables was obtained by subtracting the value obtained for cholesterol from that of total fat.

For the determination of lecithin the method of Kober and Egerer¹⁶ for phosphorus, as modified by Bloor⁴ for blood lecithin, was used. In regard to the lecithin determinations considerable difficulty was encountered in preparing a satisfactory strychnine molybdate reagent for precipitating the phosphorus. After testing out the purity of different chemicals involved in preparing the reagent it was found that the trouble was due to the sodium molybdate. Kober and Egerer had found Merck's sodium molybdate satisfactory, but on account of the war they had to prepare this substance themselves from molybdic acid and sodium hydroxide. Using the ordinary c.p. stick sodium hydroxide and pure molybdic acid did not produce a satisfactory sodium molybdate, but substituting Merck's sodium hydroxide (in lumps) prepared from sodium a satisfactory sodium molybdate for preparing the strychnine molybdate reagent was obtained. It is possible that the difficulty of obtaining a pure sodium molybdate is due to the sodium hydroxide containing some phosphorus as an impurity.

Experiments were carried out on two dogs.

Experiment 1.—Dog 1 was a healthy male weighing 9.5 kg. It was fed 10 cc. olive oil at 10.20 a.m. At 10.25 a.m. the first sample of blood was taken. After taking a 3 cc. sample for whole blood, the remainder was centrifuged for 10 minutes at 2,000 revolutions per minute. The corpuscles were found to be 52 per cent. The plasma was clear and yellow in color. A 3 cc. sample was taken for analysis. The other samples were as follows.

Sample.		Corpuscles.	Plasma.
	p.m.	per cent	
2	12.25.....	52.2	Cloudy.
3	2.25.....	51.6	" white.
4	4.35.....	48.6	Milky.
5	6.20.....	48.	Cloudy.

Experiment 2.—Dog 1 as before, weight 9.5 kg. Fed 60 cc. olive oil at 10.33 a.m. First blood sample 10.38 a.m. Corpuscles 49.4 per cent. Plasma clear.

¹³ Bloor and Knudson, *J. Biol. Chem.*, 1917, xxix, 7.

¹⁴ Bloor, *J. Biol. Chem.*, 1914, xvii, 384.

¹⁶ Kober, P. A., and Egerer, G., *J. Am. Chem. Soc.*, 1915, xxxvii, 2373.

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Sample.	p.m.	Corpuscles. per cent	Plasma.
2	12.30.....	47.	Cloudy, white
3	2.40.....	48.7	Milky.
4	4.45.....	47.	"
5	6.30.....	47.9	"

Experiment 3.—Dog 1 as above, weight 10.3 kg. Fed 65 cc. olive oil at 10.48 a.m. First blood sample at 10.53 a.m. Corpuscles 47.5 per cent. Plasma clear.

Sample.	p.m.	Corpuscles. per cent	Plasma.
2	12.52.....	52.6	Cloudy, white.
3	2.53.....	53.	" greater than No. 2.
4	4.54.....	52.8	Milky.
5	6.53.....	48.5	" slightly.

Experiment 4.—Dog 2 was a healthy male weighing 8 kg. Fed 50 cc. olive oil at 10.05 a.m. First blood sample at 10.10 a.m. Corpuscles 52 per cent. Plasma clear.

Sample.	p.m.	Corpuscles. per cent	Plasma.
2	12.10.....	53.	White cloudiness.
3	2.15.....	53.	Milky.
4	4.15.....	53.	Slightly milky.
5	6.15.....	49.4	Milky.

Experiment 5.—Dog 2 as above, weight 8.3 kg. Fed 60 cc. olive oil at 9.25 a.m. First sample at 9.30 a.m. Corpuscles 50 per cent. Plasma clear.

Sample.		Corpuscles. per cent	Plasma.
2	11.35 a.m.....	52.	White cloudiness.
3	1.40 p.m.....	51.5	Milky.
4	3.30 ".....	51.3	White cloudiness.
5	5.40 ".....	50.	Almost clear.

Experiment 6.—Dog 2 as above, weight 8.6 kg. Fed 65 cc. olive oil at 9.40 a.m. First sample at 9.45 a.m. Corpuscles 52 per cent. Plasma clear.

Sample.		Corpuscles. per cent	Plasma.
2	11.45 a.m.....	50.	Cloudy.
3	1.50 p.m.....	52.	"
4	3.50 ".....	51.	Milky.
5	5.50 ".....	51.	Cloudy.

The dogs used in these experiments were previously accustomed to handling, and did not struggle during the experiments or show

other evidences of excitement, so those factors would not influence these results.

The analytical results are given in Table I. Direct deter-

TABLE I.

Cholesterol and Cholesterol Esters during Fat Absorption. Mg. per 100 Cc.

No. of experiment.	Time.	Whole blood.			Blood plasma.			Corpuscles.		
		Total.	As ester.		Total.	As ester.		Total.	As ester.	
		mg.	mg.	per cent	mg.	mg.	per cent	mg.	mg.	per cent
1	Before.	221	57	25.8	227	110	48.5	215	7	3.2
	2 hrs.	221	80	36.2	231	129	55.8	213	34	15.9
	4 "	208	71	34.1	217	125	57.7	200	20	10.
	6 "	210	83	39.5	213	135	63.4	208	28	13.4
	8 "	210	64	30.4	217	121	55.7	202	2	1.
2	Before.	220	57	25.9	210	111	52.9	230	2	0.9
	2 hrs.	217	74	34.1	208	126	60.6	228	15	6.5
	4 "	211	71	33.7	196	110	56.2	226	32	14.1
	6 "	219	90	41.1	*					
	8 "	208	87	41.7	197	140	71.2	222	29	13.
3	Before.	195	56	28.7	179	98	54.7	212	9	4.2
	2 hrs.	200	55	27.5	188	106	56.5	211	8	3.7
	4 "	201	69	34.3	182	105	57.8	218	37	16.9
	6 "	198	74	37.3	180	117	65.7	214	36	16.8
	8 "	203	60	29.5	177	96	54.2	231	22	9.5
4	Before.	196	51	26.	180	100	55.6	211	6	2.7
	2 hrs.	200	70	35.	176	105	59.1	213	39	18.3
	4 "	188	74	39.3	173	123	71.1	202	49	24.2
	6 "	197	70	35.5	174	104	59.8	217	40	18.4
	8 "	196	72	36.7	192	113	58.9	200	30	15.
5	Before.	206	55	26.6	206	105	50.9	206	5	2.4
	2 hrs.	205	76	37.	206	111	53.8	204	43	21.
	4 "	200	82	41.	210	118	56.2	195	48	24.6
	6 "	204	80	39.2	203	115	56.7	204	47	23.
	8 "	211	84	39.8	201	123	61.2	220	64	29.
6	Before.	213	54	25.3	193	110	57.	231	2	0.8
	2 hrs.	219	59	26.9	204	117	57.5	234	1	0.4
	4 "	221	58	26.2	208	121	58.2	233	0	0.
	6 "	206	83	40.2	195	126	64.7	217	42	19.3
	8 "	220	62	27.3	210	122	58.2	229	4	1.7

* This specimen of blood plasma was lost in making up to volume.

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TABLE I—Concluded.

Total Fatty Acids and Lecithin in the Blood during Fat Absorption. Gm. per 100 Cc.

No. of experiment.	Time.	Total fatty acids.			Lecithin.		
		Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.
		gm.	gm.	gm.	gm.	gm.	gm.
1	Before.	0.681	0.730	0.637	0.292	0.297	0.287
	2 hrs.	1.124	1.065	1.197	0.325	0.312	0.338
	4 "	1.086	1.015	1.169	0.374	0.331	0.415
	6 "	0.982	0.903	1.067	0.432	0.361	0.508
	8 "	0.983	0.883	1.085	0.410	0.334	0.492
2	Before.	0.756	0.745	0.766	0.263	0.300	0.227
	2 hrs.	0.869	1.010	0.708	0.266	0.290	0.241
	4 "	1.096	1.076	1.115	0.346	0.337	0.355
	6 "	1.131	†		0.365	†	
	8 "	1.059	1.053	1.064	0.356	0.300	0.418
3	Before.	0.686	0.608	0.788	0.279	0.305	0.251
	2 hrs.	0.788	0.704	0.847	0.290	0.325	0.259
	4 "	1.251	0.842	1.615	0.353	0.349	0.397
	6 "	1.118	1.055	1.173	0.398	0.340	0.451
	8 "	1.087	0.969	1.214	0.285	0.311	0.258
4	Before.	0.679	0.701	0.650	0.277	0.332	0.233
	2 hrs.	0.921	0.824	1.007	0.309	0.370	0.255
	4 "	1.236	0.988	1.454	0.347	0.398	0.302
	6 "	1.182	0.942	1.392	0.364	0.346	0.379
	8 "	1.037	0.880	1.154	0.294	0.346	0.242
5	Before.	0.721	0.665	0.778	0.341	0.312	0.350
	2 hrs.	1.079	0.764	1.372	0.336	0.348	0.325
	4 "	1.100	0.962	1.228	0.393	0.336	0.447
	6 "	0.993	0.863	1.115	0.412	0.354	0.468
	8 "	0.913	0.789	1.038	0.333	0.366	0.300
6	Before.	0.754	0.717	0.789	0.330	0.324	0.337
	2 hrs.	1.071	0.800	1.342	0.374	0.290	0.458
	4 "	0.941	0.837	1.035	0.374	0.290	0.451
	6 "	1.428	0.900	1.932	0.411	0.322	0.497
	8 "	0.856	0.815	0.962	0.418	0.359	0.474

† Specimen lost while making up to volume.

minations were made only on the whole blood and plasma from which, knowing the percentage of corpuscles in the blood, the composition of the corpuscles was calculated.

RESULTS AND DISCUSSION.

Cholesterol (Total).—The changes in total cholesterol are slight and inconstant and in most cases remain the same throughout the experiments, thus confirming the results reported by Bloor.⁴

Cholesterol Esters.—The values for cholesterol esters present some interesting relations. In all the experiments the analyses before fat absorption show that the cholesterol as esters bears a constant relation to the total cholesterol. In the whole blood it is found that from 25.3 to 28.7 per cent is combined as esters and in the plasma from 48.5 to 57 per cent. The values for the corpuscles, being determined indirectly from the whole blood and plasma, would not as a consequence be as reliable; however, it is noteworthy that the amount of ester is very low, varying from 0.8 to 4.2 per cent. It may be possible that determinations made directly and with methods yielding absolute values would show no combined cholesterol in the corpuscles as is generally believed now.

During the absorption of fat it is found that the amount of cholesterol as esters increases in all these experiments.

In the whole blood the greatest increase is noted in Experiment 2 where cholesterol as esters increases from 57 mg. to 90 mg. or 57.8 per cent. In the other experiments the increase varies from 32 to 53 per cent with an average increase for all experiments of 47.4 per cent. In the plasma the increases are not so great and the greatest increase is in Experiment 4, being 23 per cent, the others varying from 14.5 to 22 per cent. On the other hand, the increases in the corpuscles are most marked. If one considers the calculated values for esters in the corpuscles before fat absorption as correct, then after absorption the increases are very striking, varying from 300 per cent in Experiment 1, to 1,170 per cent in Experiment 5 and even 2,000 per cent in Experiment 6.

The increase during fat absorption is infinitely greater if one

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considers the normal amount of esters in the corpuscles as negligible.

The fact that cholesterol esters increase during fat absorption bears out the assumption that cholesterol takes an active part in the metabolism of fatty acids. The maximum increase of cholesterol esters during fat absorption occurs in most experiments about the 6th hour after feeding, while for the total fatty acids it occurs about the 4th hour.

It is interesting to note also that the cholesterol esters bear a somewhat general constant relation to the total fatty acids and to the lecithin, as shown in Table II.

TABLE II.
Relation between Total Fatty Acids and Cholesterol Esters and between Lecithin and Cholesterol Esters in the Whole Blood.

Period.	Total fatty acids. Cholesterol esters.						Lecithin. Cholesterol esters.					
	Experiment.						Experiment.					
	1	2	3	4	5	6	1	2	3	4	5	6
1	11.9	13.2	12.2	13.3	13.1	13.9	5.1	4.6	5.0	5.4	6.2	6.1
2	14.1	12.2	14.3	13.2	14.1	18.2	4.1	3.7	5.3	4.4	4.4	6.3
3	15.4	15.4	18.1	16.7	13.4	16.2	5.3	4.7	5.1	4.7	4.8	6.4
4	11.7	12.6	15.1	16.8	12.5	17.2	6.1	4.1	5.4	5.2	5.1	4.8
5	16.9	12.6	18.1	14.4	9.7	16.	6.4	4.1	4.8	4.1	4.5	6.4

It is hardly to be expected that an exactly constant relation could exist as the cholesterol esters reach their maximum on the whole a little later than the total fatty acids. The relation between lecithin and cholesterol esters seems to be more constant than relation between total fatty acids and cholesterol esters. This constancy of relation between lecithin and cholesterol esters might be taken as evidence that cholesterol esters are one of the intermediate stages in fat metabolism as well as lecithin. Just what function the formation of cholesterol esters plays in fat metabolism is difficult to tell at present.

Total Fatty Acids.—The fatty acids show the ordinary increases which have been noted by several investigators during fat absorp-

tion.^{17-19,4} In the whole blood the greatest increase was 89 per cent in Experiment 6 and it varied in the other experiments from 49 to 82 per cent. In the plasma the increases are less, varying from 25 to 73 per cent. The most marked increases are noted in the corpuscles varying from 48 to 145 per cent. These results bear out the findings of Bloor⁴ and Munk²⁰ who likewise noted that fat of corpuscles is increased most during fat absorption and assumed that the corpuscles actively absorb the fat from the plasma.

Lecithin.—The results for lecithin confirm the findings of Bloor.⁴ In all the experiments lecithin is found to increase in the blood during fat absorption. In the whole blood the increases are from 26 to 48 per cent, in the plasma increases are much less, varying from 9 to 21 per cent, while in the corpuscles they are most marked varying from 42 to 84 per cent.

In both the lecithin formation and cholesterol esters it is to be noted that the greatest increases occur in the blood corpuscles. This would indicate that the blood corpuscles take a very active part in the absorption and assimilation of fat. The corpuscles take up as much fat as possible and transform it into cholesterol esters and lecithin which are regarded as intermediate stages of fat metabolism.

SUMMARY.

Determinations of the balance between cholesterol and cholesterol esters along with determinations of total fat and lecithin have been made in the whole blood and plasma (and by calculation in the corpuscles) during a series of fat absorption experiments with the following results.

1. The quantity of cholesterol showed no constant change agreeing with earlier investigations.
2. The cholesterol esters increased in both plasma and corpuscles but the increase is most marked in the corpuscles.
3. The total fatty acids and lecithin likewise increased in plasma and corpuscles but the increase is greater in the corpuscles.

¹⁷ Terroine, *J. physiol. et path. gen.*, 1914, xvi, 386.

¹⁸ Mendel, L. B., and Baumann, E. J., *J. Biol. Chem.*, 1915, xxii, 165.

¹⁹ Bloor, *J. Biol. Chem.*, 1915, xxiii, 317.

²⁰ Munk, I., and Friedenthal, H., *Zentr. Physiol.*, 1901-02, xv, 297.

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4. A fairly constant relationship between total fatty acids and cholesterol esters and between lecithin and cholesterol esters was noted in the whole blood.

5. The greater increases of cholesterol esters and lecithin in the blood corpuscles, along with the greater amount of fatty acids, would indicate that the blood corpuscles play a very active part in fat metabolism.

A BIOLOGICAL ANALYSIS OF PELLAGRA-PRODUCING DIETS.

III. THE VALUES OF SOME SEED PROTEINS FOR MAINTENANCE.

By E. V. McCOLLUM AND N. SIMMONDS.

(From the Laboratory of Agricultural Chemistry of the University of Wisconsin, Madison.)

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When an animal is fed solely upon a single kind of seed, regardless of the kind of plant from which it is derived, loss of weight ensues and death is certain to supervene within a few months. Due to this fact it has not been possible in the past to determine experimentally the comparative biological values of the proteins of one variety of seed as contrasted with those of another. During the past 4 years we have in this laboratory developed experimental methods which make possible such a study. It depends upon the full appreciation of (1) the deficiency of all seeds in certain inorganic elements (calcium, sodium, and chlorine), and that these must be added before it is possible for an animal to make optimum utilization of the proteins of its food, (2) most of the seeds of plants are likewise deficient in the unidentified dietary essential fat-soluble A, which is found abundantly in the fats of milk and eggs and in leaves and a few seeds. When the seeds are improved from the dietary standpoint in these respects, the limiting factor in determining their biological values is the protein.

When a seed is fed supplemented with both salts (NaCl and CaCO_3) and butter fat (fat-soluble A), the limiting factor is then the quality of the protein. In order to determine the relative value of the total protein as compared with other seeds, the following modifications of this mixture are made: (a) a pure carbohydrate is added to produce a low protein mixture; (b) the mixture is fed without any carbohydrate addition; (c) the mixture is fed with a protein preparation from the seed employed so as to raise the plane of protein intake to higher levels. The results

of feeding rations so planned show the relative value of the total protein of the seed as compared with that of other seeds.

The interpretation of the data depends upon the plane of protein intake from each seed which is necessary to just maintain an animal without loss of body weight, and the plane which will just support growth at some particular rate, *e.g.*, half or full normal as estimated from the curve of normal expectation for the species. We have discussed in previous papers the rates of growth obtained on diets in which the protein was fed at about 8 per cent of the food mixture or at higher levels (1). In the present communication we present data which show the minimum protein intake which suffices for maintenance when derived from the seeds of wheat, maize, oat, rice (polished), millet seed, flaxseed, bean, and pea and, for comparison, an experiment in which the sole protein (6 per cent) of the diet was from the alfalfa leaf.

A question of the greatest importance receives a fairly definite answer from these experiments in which we closely approximated the protein minimum for maintenance using a single naturally occurring food of vegetable origin as the sole source of protein, and continued the experimental trials long enough to demonstrate their physiological effects. We have here experiments with rations so constituted that the *effects of a low protein diet* without complications of any kind are visible.¹ Details concerning the condition of the animals are given in the legends to the charts. One fact was evident in all the lots of animals observed, *viz.*, the marked tendency to rapid loss of weight and failure to maintain body weight in the case of most of the animals, although a few were able to do so, for a considerable period. This can be interpreted only to mean, we believe, that the vitality is greatly lowered by a diet, otherwise adequate, but near the physiological minimum in its protein content.

¹ Some work with diets of this nature in which milk and wheat proteins were employed has been already reported by McCollum and Davis (*J. Biol. Chem.*, 1915, xx, 415). Osborne and Mendel have likewise fed "protein-free milk" with purified proteins at low planes of intake (*J. Biol. Chem.*, 1915, xx, 351). In their rations the nitrogen in uncharacterized forms found in "protein-free milk" constituted 7 to 63 per cent of the total nitrogen of the diet so that without further study their results *cannot* be interpreted as showing the relative biological values of the *pure* proteins employed.

The data on maize, wheat, oats, and flaxseed presented in the charts support the conclusions arrived at in this laboratory in experiments with swine confined to metabolism cages and fed a single kind of seed as a source of protein (2). Nitrogen balances were kept on the animals during periods of 30 to 40 days. The protein mixtures of the wheat, oat, and maize kernels differ but little in their biological values. With swine it was found that wheat, maize, and oat proteins could be retained for growth to the extent of 20, 23, and 26 per cent respectively of the amount ingested. With rats 6 per cent of wheat or maize proteins just suffice to maintain the animals in body weight, while 4 per cent of oat proteins has maintained them in distinctly better condition as judged by their appearance than did wheat and maize proteins at 6 per cent of the food mixture.

There may seem in these observations on oat proteins to be an apparent contradiction to the results we have already published concerning the dietary properties of the oat kernel (3). A high consumption of rolled oats (uncooked), we concluded, exerted an injurious effect upon growing rats. This we attributed provisionally to the tendency to form pasty feces which were difficult of elimination. We pointed out that this depressing factor made uncertain our interpretation of the amount of growth secured with various planes of oat proteins. In other words we suspected that the oat proteins might be of higher biological value than the results seemed to indicate. The results of feeding oat proteins at still lower levels for maintenance records tend to confirm us in this belief. With the low intake of oats the injurious effects would of course decrease and permit the animals to manifest in appearance and health the beneficial effects of a satisfactory amino-acid mixture in the diet.

Flaxseed proteins appear in these maintenance experiments with rats to have a still lower biological value when fed as the sole source of protein, than have the proteins of the wheat, oat, or maize kernels. This again is in harmony with our former results with swine in which nitrogen balance records indicated that this species can retain for growth only 16 to 17 per cent of the nitrogen ingested as flaxseed proteins. It requires about 8 per cent of flaxseed proteins in the food mixture to maintain a grown rat.

An excellent supplementary relationship exists between the proteins of the flaxseed and the maize kernel (2).

One of the most surprising results of this investigation is the value which it assigns to the proteins of polished rice as compared with the other cereal grains. The results of other investigators had led us to expect that rice proteins should show an extraordinary biological value. Karl Thomas (4), working upon himself as a subject, attempted to find the lowest intake of nitrogen, as protein, which, derived in turn from a single one of a number of more common foodstuffs, would maintain him in nitrogen equilibrium. He expressed his results as the "biological values" by which he indicated the per cent of the food protein which could be converted into body protein. Thomas assigned the following relative values to four seeds which are remarkable for the contrast they show with our observations:

	Thomas' values. per cent
Rice.....	88.3
Pea.....	55.7
Wheat.....	39.5
Maize.....	29.5

We have recently pointed out (5) that young rats fail to grow on a diet which is entirely satisfactory except that its sole protein content of 10 per cent was derived entirely from cooked peas plus gelatin, yet they responded with rapid growth when pure protein (casein) was substituted for the gelatin. Poor quality of the pea proteins was therefore responsible for their failure to grow. 9 per cent of either wheat proteins or of maize proteins support growth at about half the normal rate (1) and have therefore about the same value. A slightly better growth with wheat is probably due to the lower digestibility of maize proteins. Pea proteins are therefore distinctly inferior to either the wheat or maize proteins. These observations on growth are entirely in harmony with the maintenance experiments shown in Charts 1, 2, and 3 for wheat and maize and in Chart 12, which shows maintenance on 11.5 per cent of pea and of bean proteins. Several of these rats had acquired but 60 to 70 per cent of their growth but they were unable to grow further in any instance.

The work of Thomas has been frequently cited as authoritative. Indeed Osborne, Van Slyke, Leavenworth, and Vinograd (6)

after observing the lysine content of mixtures approximating the character of the proteins of the maize, wheat, and rice kernels to be 0.97, 1.58, and 4.26 per cent, respectively, called attention to the remarkable correspondence between the lysine content of the proteins of these seeds and the biological values of the same proteins as determined by Thomas. This they regarded as a strong argument in support of the idea that lysine is in all these seeds the essential amino-acid which is present in smallest amount and is therefore the limiting factor in determining the biological value of the proteins in each.

Our results, when checked, both regarding maintenance at low planes and growth at higher planes of protein intake in the rat, and by growth studies with young swine, are so absolutely at variance with those of Thomas, that we are forced to the conclusion that some error must have occurred in his work. We have recently shown that lysine cannot possibly be the limiting amino-acid in either the maize or oat kernel (7).

So far as *chemical* methods have been able to show, the proteins of the bean and pea contain all the known amino-acids necessary for nutrition and in no unsymmetrical distribution as in the case of wheat and maize proteins, yet the legume proteins have only about one-half the value of those of wheat and maize. Such experiences as these emphasize what must be conceded by one who reflects upon the problem, *viz.*; *that there is entirely too great a tendency to put faith in the data derived from a chemical analysis as an indication of the value of the proteins in animal nutrition.*

Millet seed, which, together with flaxseed, we have shown to contain a much greater amount of fat-soluble A than is found in the ordinary cereal grains, differs very greatly from flaxseed in the value of its proteins when fed as the sole source of nitrogen. Millet seed proteins possess a somewhat higher value for maintenance than those of any other seed in the list reported in this paper, except oats.

Pea and bean proteins possess about the same biological values when fed alone, but the fact that the value of bean proteins is enhanced by oat proteins whereas pea proteins are not (5), shows that the essential amino-acid which forms the limiting factor is not the same in these two legume seeds. About 11 per cent of proteins from either of these seeds is required for maintenance.

Cottonseed proteins are of relatively good quality as indicated by the maintenance of body weight in all rats fed a ration whose 6 per cent protein content was derived from this source.

As compared with the seed proteins employed in the present experiments, the nitrogen of alfalfa leaves shows, when fed as the sole source of protein, no superiority. Without more extensive data, it is undesirable to speculate regarding the biological value of the protein and non-protein nitrogen of this or other leaves.

In considering the data presented above, it should not be lost sight of that in all cases such additions were made to the seeds as made good their dietary deficiencies other than protein. The results are not to be interpreted as applying to the seeds themselves but to the seeds with the additions noted.

A few words should be said regarding the application of this data to the interpretation of the dietary factors operating to produce pellagra. From single seeds the plane of protein intake, which is necessary for maintenance of body weight in grown or nearly grown rats, when all other dietary factors are properly adjusted, varies from 4 to 6 per cent in the case of millet seed, oat, wheat, maize, rice, flaxseed, and cottonseed to about 11 per cent in the navy bean and pea. Millet seed and oat proteins are distinctly better than those of the other seeds named. We are conscious that the objection may be raised that we are not in these experiments comparing the relative values of the total protein of the seeds, but rather that portion which is digested and absorbed. Nothing is gained by applying to the data a digestibility factor and comparing "digestible protein." The apparent digestibility of a food depends not alone upon the extent to which the protein of the food is capable of being hydrolyzed by enzymatic action, but by the presence of large or small amounts of indigestible material which not only may prevent access of the secretions to the food but likewise hold from absorption the digested material (8). As we express the results we are showing the comparative values of what the animal gets from each of these seeds when it is introduced into its alimentary tract under specified conditions.

For wheat (1) and maize (1), we know furthermore that the maintenance requirement for protein must at least be doubled before growth can be approximately normal in the young. This is likewise true for milk proteins. Maintenance is secured over a

period of 5 or 6 months (9) on milk proteins to the extent of 3 per cent and normal growth is first secured with 6 per cent of milk protein in the diet. On this plane of milk protein intake growth became retarded before the full adult size was attained. Such observations as these together with the early appearance of the signs of old age in rats whose protein supply was just sufficient to enable them to complete their growth at the usual rate (10), lead us to the belief that a moderate excess of protein is necessary over the amount required to maintain nitrogen equilibrium during a long period, if the optimum well-being is to be attained.

The diet employed by Goldberger (11) for the production of experimental pellagra in man consisted of dishes prepared from polished rice, patent flour, corn grits, pork fat, cornstarch, syrup, sweet potatoes, cabbage, collards, and turnip greens. The protein consumed was probably not far from 8 per cent of the food mixture or 30 per cent above the indispensable minimum on which a few exceptionally vigorous rats in middle life could live about one-third to one-half of their normal expectation of life, maintaining their weight over most of this period. Whether this constitutes a safe margin is of course an open question until actually tested experimentally. We shall offer definite evidence on this point in a subsequent paper. It is certain, however, that the protein content of Goldberger's diet, derived almost wholly from seeds, is too low to support normal growth, and is close to the point where the resistance powers of the adult will be decreased.

A point deserving of special comment is brought out by the records of Lot 987, Chart 8. These animals were confined to a diet which contained but 4 per cent of protein and derived all of its fat-soluble A from 33 per cent of millet seed. This amount of millet seed suffices, when combined with pure casein, a suitable salt mixture, and dextrin, to induce good growth and maintain normal health over a long period (5) (Chart 9, Rat 716). In other words it supplied a sufficient amount of both the dietary A and B to support physiological well-being. The remaining dietary factors were fairly satisfactorily constituted. Numerous young were produced but without the addition of more fat-soluble A none could be reared (9).

In the case of Lot 987, however, the animals all became blind after 60 to 70 days of confinement to this diet. Xerophthalmia and blindness are the most striking visible symptoms resulting from a deficiency of the dietary factor fat-soluble A. We are, therefore, confronted with the paradoxical evidence that what is enough of this substance in one case is not enough in another. We have previously met with similar situations involving other components of the diet; *e.g.*, protein (12, p. 112). The explanation of these results appears to be that there is in reality no quantity of protein, fat-soluble A, or other constituent of the diet which can be designated as the physiological minimum, without the biological values of every other dietary factor being also stated. *The least amount of butter fat which will suffice to support growth when the diet is otherwise of good constitution, will not be adequate in another case in which the quality of one or another factor is of a low order. This idea should be kept clearly in mind in interpreting the etiology of pellagra, in cases where several dietary factors fall below the optimum.*

Liberal consumption, prompt digestion and absorption and prompt evacuation of undigested residues from the intestine before extensive absorption of products of bacterial decomposition of proteins can take place are the optimum conditions for the maintenance of vigor and the characteristics of youth.

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Salt mixture 318.

	<i>gm.</i>
NaCl.....	1.40
K ₂ HPO ₄	2.531
K citrate, H ₂ O.....	0.710
CaSO ₄	0.578
Ca lactate.....	7.058

Salt mixture 308.

	<i>gm.</i>
NaCl.....	9.5
K ₂ HPO ₄	1.211
CaH ₄ (PO ₄) ₂ ·H ₂ O.....	0.256
Fe citrate.....	0.194
Ca lactate.....	2.994

Salt mixture 185.

	<i>gm.</i>
NaCl.....	0.173
MgSO ₄ (anhydrous).....	0.266
NaH ₂ PO ₄ ·H ₂ O.....	0.347
K ₂ HPO ₄	0.954
CaH ₄ (PO ₄) ₂ ·H ₂ O.....	0.540
Fe citrate.....	0.118
Ca lactate.....	1.300

Salt mixture 500.

	<i>gm.</i>
NaCl.....	0.5148
CaCl ₂	0.2569
K ₂ HPO ₄	0.3113
K citrate.....	0.5562
Ca lactate.....	2.878

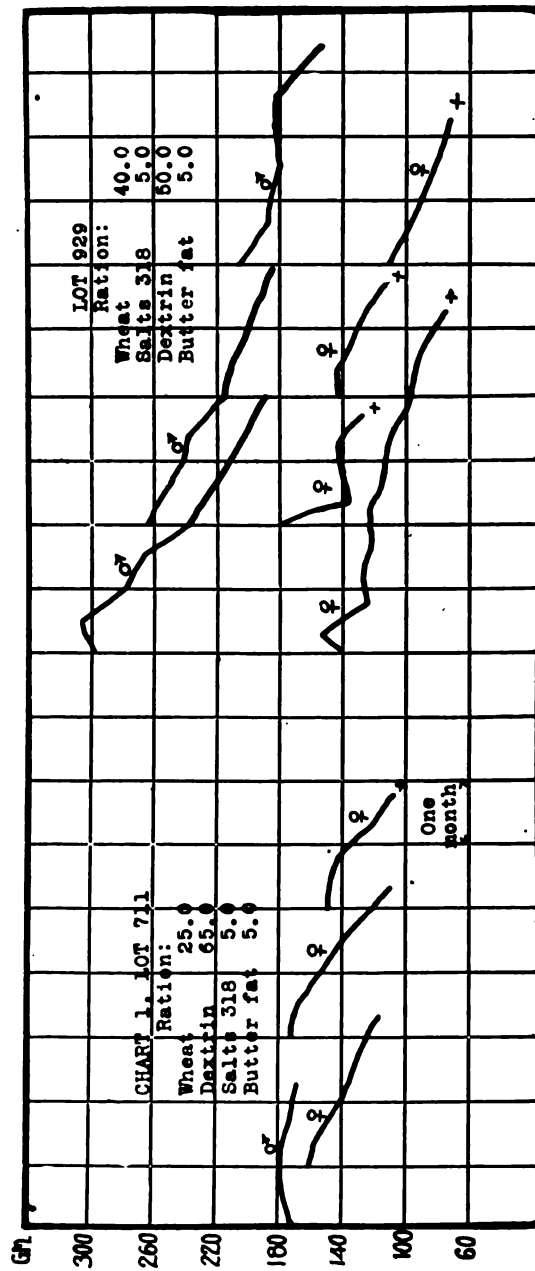


CHART 1. Lot 711 shows steady decline in body weight in rats whose ration was satisfactory in all its parts except the protein factor. The sole protein supply in this food mixture amounted to only 2.5 per cent and was derived from the whole wheat kernel.

Lot 929 whose ration was closely similar in all respects to that of Lot 711 except that the protein content which was derived entirely from wheat, made 4 per cent of the food mixture. The great variation in the span of life of individual rats on these low protein rations is of interest. All were in good nutritive condition at the beginning of the experimental period, and if properly nourished were capable of living 1 to 2 years longer.

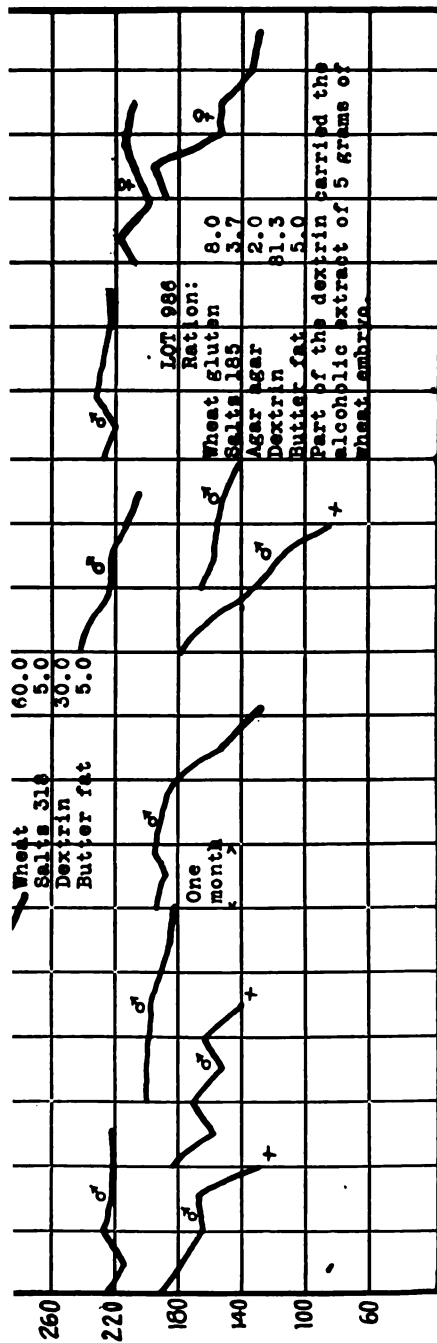


CHART 2. Lot 964 whose ration was properly adjusted in all but the protein factor which was below the optimum, shows that some individuals can maintain body weight on wheat protein at 6 per cent of the food mixture. The vitality is lowered by reducing the plane of protein intake to a point near the maintenance level, as is shown by the fact that of a group of grown rats, all in fine condition, some cannot maintain themselves while others more vigorous show no immediate signs of failure. Maintenance of body weight during 2 months or more does not mean that these diets are safe even for the adult rat.

The records of Lot 986 show that 6 per cent of wheat gluten proteins maintains without loss of body weight about the same proportion of vigorous adult rats as does 6 per cent of the entire wheat proteins.

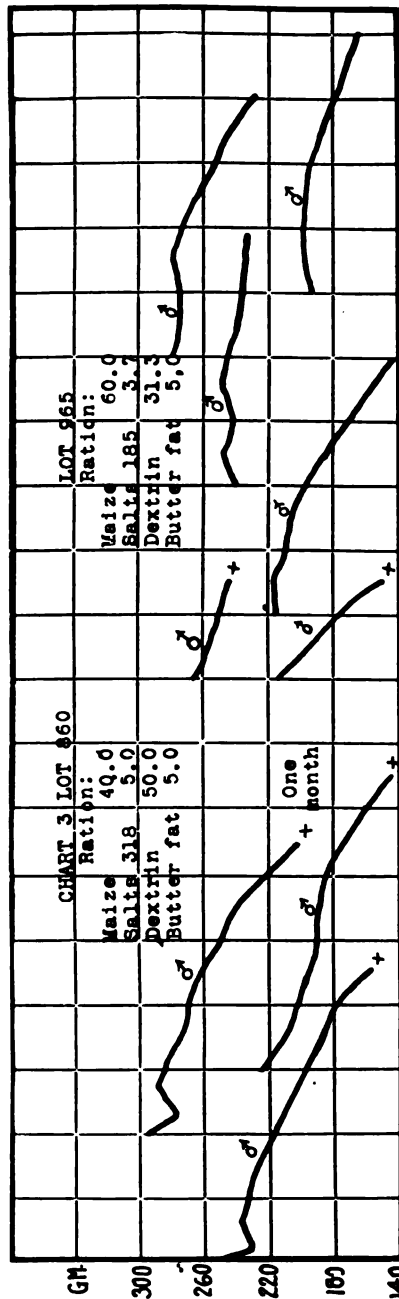


CHART 3. Lots 860 and 965 illustrate the fact that all rats steadily fail on 4 per cent of maize proteins, the other factors of diet being properly adjusted, while a few can maintain body weight over more than 2 months on 6 per cent of the maize proteins. The similarity in the biological values of wheat and maize kernel proteins is very marked.

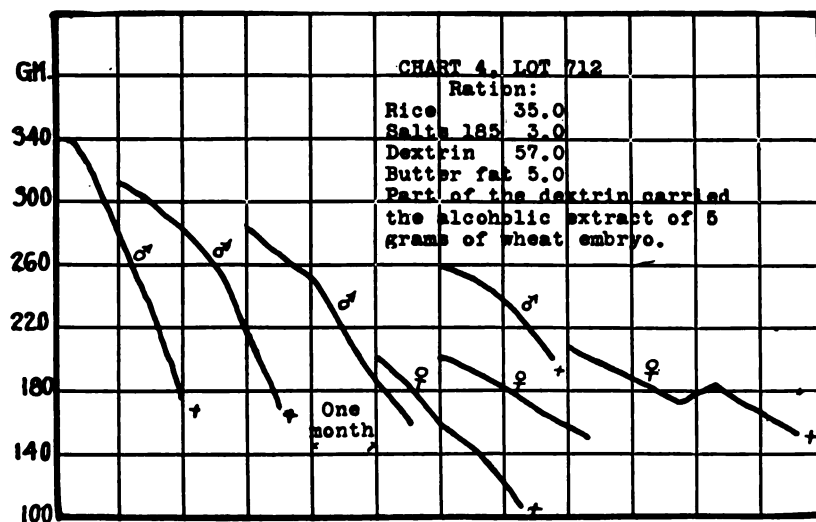


CHART 4. Judging from the work of Thomas (4) we had anticipated an unusually high value for the proteins of rice. The ration of Lot 712 which contained about 2.8 per cent of protein permitted rats to lose weight very rapidly. According to Thomas rice proteins possess more than twice the biological value of wheat proteins. We are convinced that the proteins of polished rice are of about the same quality as those of wheat and maize.

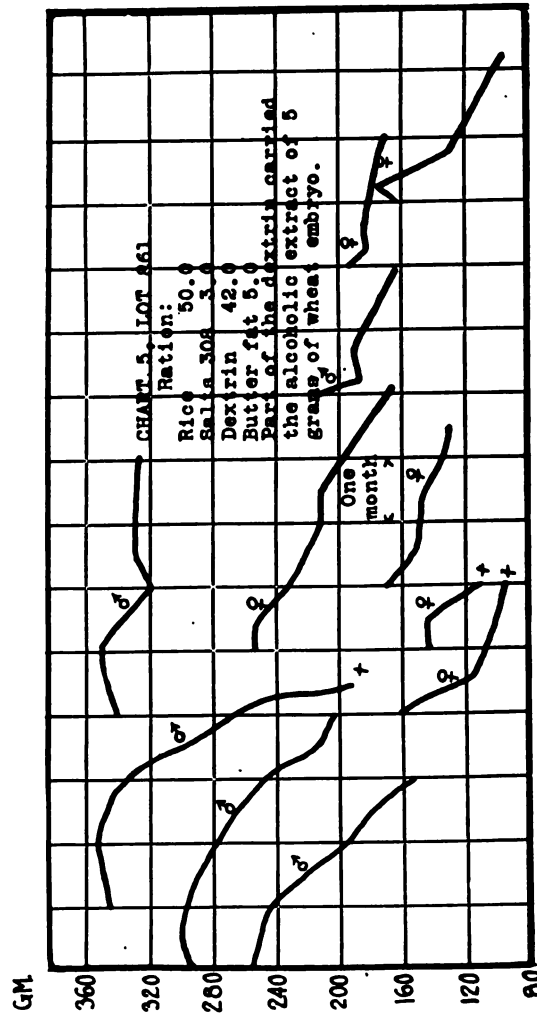


CHART 5. Lot 861 again illustrates the striking similarity of the rate of decline of adult rats on a ration otherwise satisfactory but containing only 4 per cent of protein from rice, to those on comparable rations in which 4 per cent of wheat or maize protein was supplied (Charts 1, 2, and 3). Body weight was in no case maintained on less than 6 per cent of the proteins of one of these seeds in the food mixture (Chart 6).

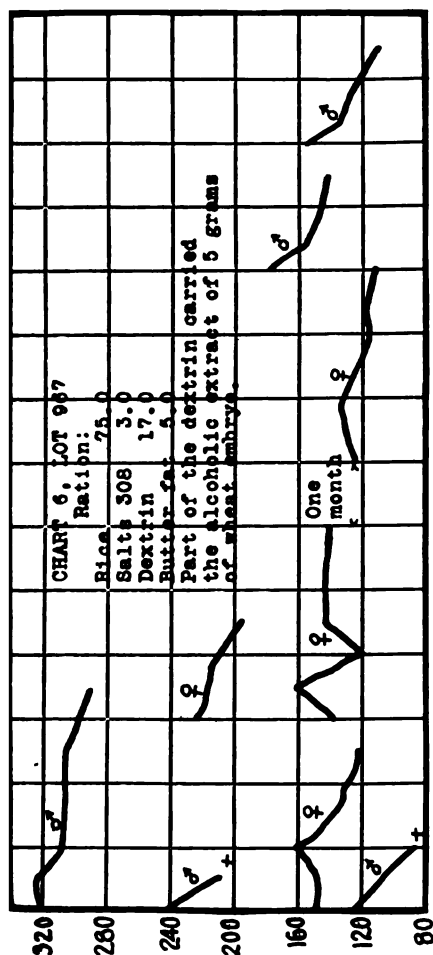


CHART 6. Lot 967. This ration contained 6 per cent of protein of polished rice. Two out of nine individuals did not lose weight during 2½ months, while all others declined steadily. Rice protein is very rich in the diamino-acid lysine. Since we have shown (7) that lysine is not the limiting factor in determining the value of the proteins of either maize or oat kernel, it becomes necessary to conclude that no generalization can be made as to the exact natures of the deficiencies of the proteins of our vegetable foods. Rice proteins like those of maize and wheat are of relatively low biological value.

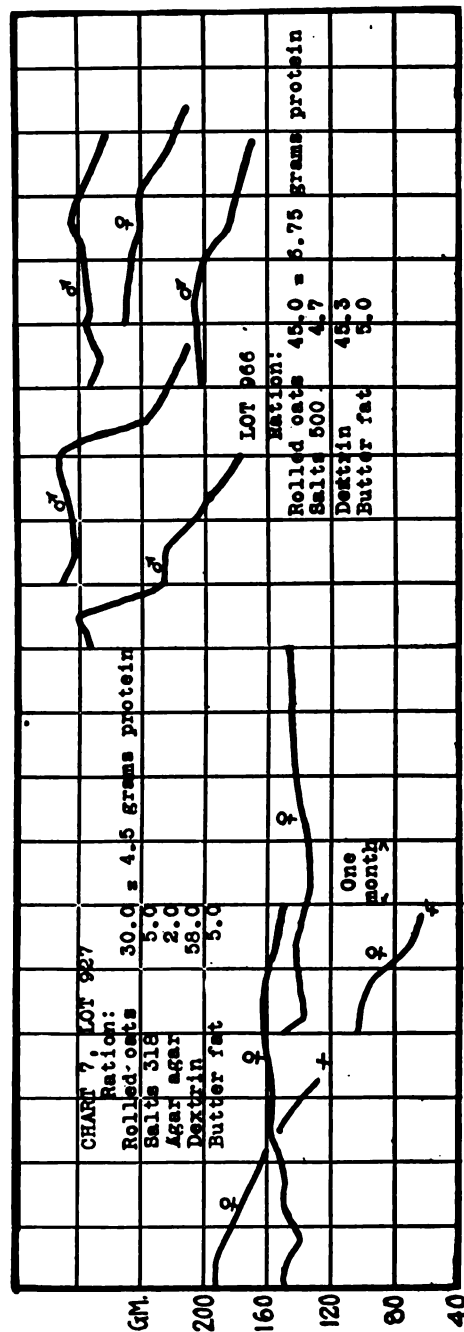


CHART 7. Lot 927 shows remarkable maintenance records in the case of two adult rats. The ration furnished but 4.5 per cent of protein, all from the oat kernel. This indicates that the oat proteins are distinctly better than those of wheat, maize, or rice. At higher planes of intake oats are injurious to the rat. It is not possible to compare oats with other seeds with accuracy as to the values of oat proteins for growth. The depressing factor in oats, whatever may be its nature (3), prevents the maximum rate of growth which the quality of the oat proteins could support.

Lot 966 whose ration supplied 6.75 per cent of oat protein and was otherwise satisfactory still permitted early decline in two of the five animals. We believe health is jeopardized by lowering the protein intake to a point near the maintenance level.

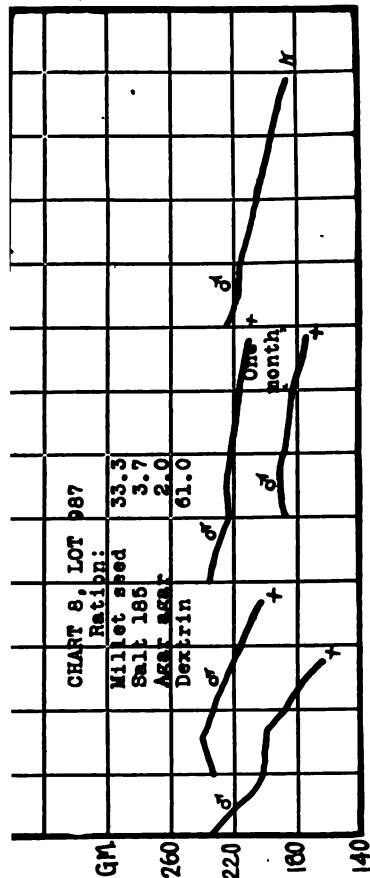


CHART 8. Lot 987. These records indicate that the proteins of millet seed are of better quality than those of wheat, maize, and rice. This diet contained but 4 per cent of protein, all derived from millet seed. The records indicate that this is for a period of 2 to 3 months at least approximately the maintenance requirement for the adult rat.

The rats in this group all became blind after about 60 days' confinement to their restricted diet. When the protein content of the diet is satisfactory rats do not go blind when furnished the amount of fat-soluble A contained in 25 per cent of millet seed. The reason for this has been discussed above.

Millet seed is extensively employed as human food in China and Russia. Where the diet is principally of vegetable origin it is especially desirable to include articles which furnish proteins of good quality. Further studies of millet seed are in progress which will show clearly whether it can be wisely introduced as a staple article of diet.

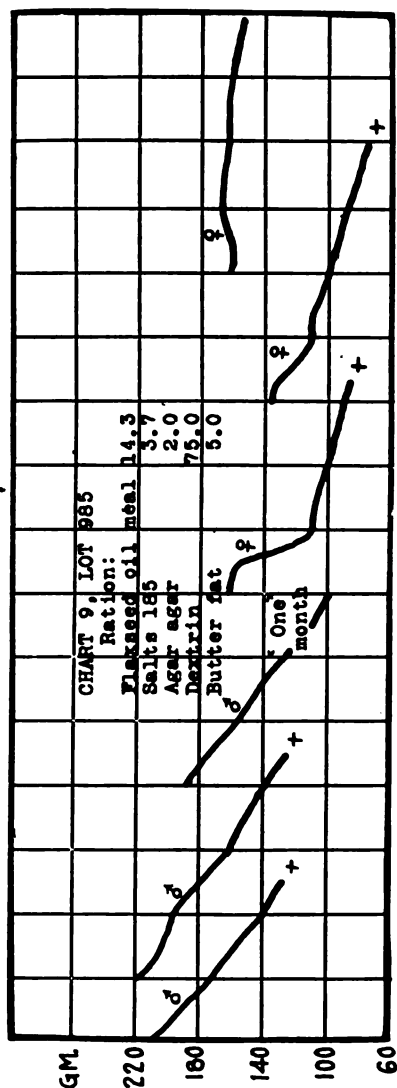


CHART 9. Lot 985 indicates that, taken as the sole source of protein, flaxseed possesses a relatively low value. Growing swine retain for the construction of body protein 20 per cent of wheat, 23 per cent of maize, and 28 per cent of oat protein. This food mixture contained 5 per cent of protein all of which was derived from flaxseed. All but one steadily lost weight and died in 3 to 4 months. All became very feeble, although they were in excellent condition at the beginning of the experiment. Natural vigor must be regarded as the cause of one being able to maintain its weight where others fail.

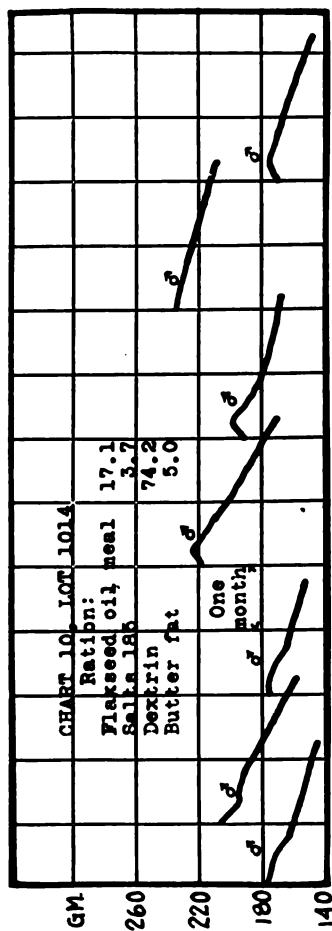


CHART 10. Lot 1014 shows slow decline of rats fed a ration which was satisfactory except in the quality and amount of its protein content. This food mixture supplied 6 per cent of flaxseed protein. At least 7 per cent of protein of the flaxseed is necessary for maintenance over a considerable period.

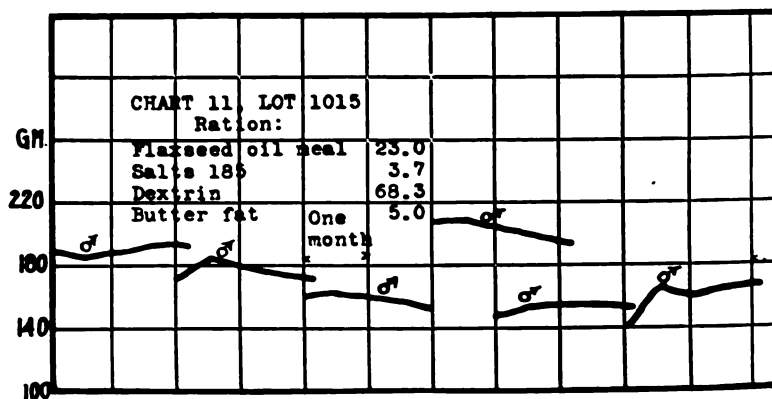


CHART 11. Lot 1015 whose ration contained 8 per cent of flaxseed protein just serves to maintain body weight. Charts 9, 10, and 11 all confirm the conclusion arrived at in experiments with growing swine, that the proteins of flaxseed are, when taken as the sole source of protein, of distinctly lower value than are those of wheat, maize, and oat kernels. (Compare Charts 1, 2, 3, 4, and 7.)

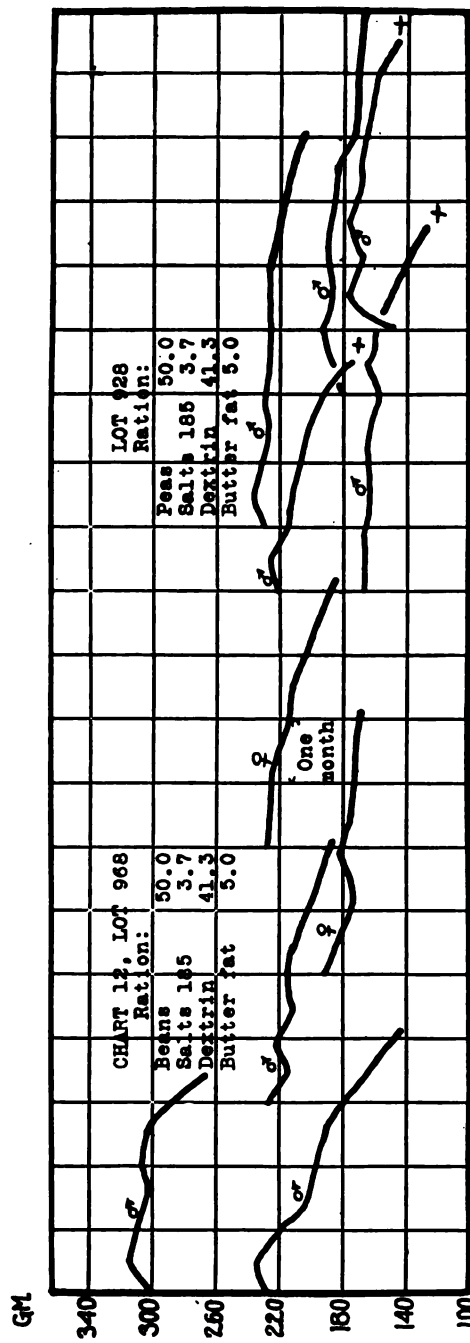


CHART 12. Lot 968 makes evident the very poor quality of the proteins of the navy bean when taken without other proteins which make good their deficiencies. We have elsewhere shown that growth is not possible on a diet furnishing 11.5 (13) per cent of bean proteins. Bean and pea proteins have almost the same biological value when each is fed as the sole source of protein (Lot 928).

Lot 928 shows maintenance only on a diet the protein of which amounted to 11.5 per cent and was all derived from peas. The rats in this group were all capable of further growth, but they were prevented from developing by the poor quality of the proteins in the ration.

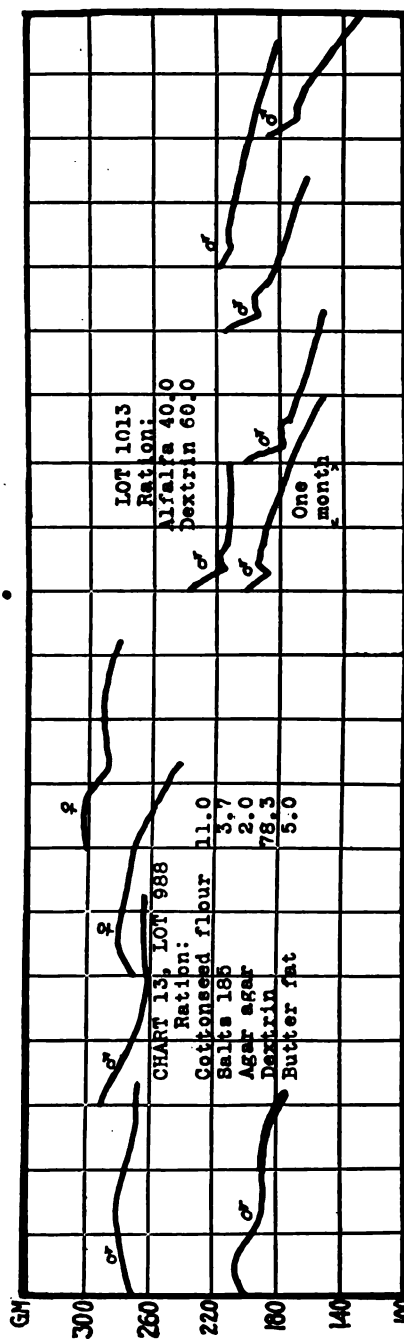


CHART 13. Lot 988 shows that the proteins of the cottonseed when fed without protein supplements have about the same value as have those of wheat or maize. The ration of this lot contained 6 per cent of proteins derived from cottonseed flour. This plane of intake just serves to maintain body weight in adult rats when the food mixture is properly constituted as respects other factors.

Lot 1013 whose sole protein supply was derived from 6 per cent of "crude protein" (N x 6.25), from alfalfa leaves steadily declined. The poor quality of the amino-acid supply was solely responsible for their failure to maintain body weight. In our experience rats cannot eat rations containing more than 40 per cent of alfalfa leaves without injury (14) so it is not readily possible to test the effects of feeding the proteins of this leaf at higher levels. The amino-acid mixture of the alfalfa leaf is therefore inferior to that yielded by any of the cereal grains. It is to be remembered that the leaf has a peculiar value in the character of its mineral content and in its high content of the unidentified dietary essential fat-soluble A. It should be reiterated **until appreciated, that a chemical analysis tells absolutely nothing as to the dietary properties of foods.** The biological analysis which we have perfected can alone yield the desired knowledge of our naturally occurring foodstuffs.

THE USE OF SOY BEAN AS FOOD.*†

BY THOMAS B. OSBORNE AND LAFAYETTE B. MENDEL.

WITH THE COOPERATION OF EDNA L. FERRY AND ALFRED J. WAKEMAN.

(From the Laboratory of the Connecticut Agricultural Experiment Station
and the Sheffield Laboratory of Physiological Chemistry in
Yale University, New Haven.)

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The demand which the international shortage of food has created for cheaply produced and easily obtainable sources of all nutrients, and particularly of suitable proteins and fats, has directed attention anew to the possibilities of the soy bean. This leguminous seed which has long been employed in the dietary of many inhabitants of the Far East has hitherto found application in this country almost solely as a suitable food for diabetics. Its use in this special application is attributable to the fact that, in contrast with other leguminous seeds, the soy bean contains traces only of starch and comparatively small quantities of those carbohydrates which are available to the human organism. Besides its introduction into diabetic cookery, the soy bean has been recommended occasionally for use in infant feeding.¹ The employment of the soy bean for silage and as a hay crop in agriculture is better known. Owing to the richness of the bean in oil, the latter is extensively expressed for commercial uses, the press cake thereby becoming available as a fertilizer or latterly as a food product.

The results of an extensive investigation² of the range of proximate composition of many varieties of soy beans grown in this country are given in the table; likewise analyses of seven commer-

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

† A preliminary account has been published in *Proc. Soc. Exp. Biol. and Med.*, 1917, xiv, 174.

¹ Ruhräh, J., *Arch. Pediat.*, 1909, xxvi, 496; *J. Am. Med. Assn.*, 1910, liv, 1664; *Arch. Pediat.*, 1911, xxviii, 841; *Am. J. Med. Sc.*, 1915, cl, 502.

² Street, J. P., and Bailey, E. M., *J. Ind. and Eng. Chem.*, 1915, vii, 853.

cial soy bean "flours" prepared from the unpressed beans. These flours contain more protein and fat and less of all the other ingredients, owing presumably to the bolting to which they are subjected.

Range of Composition of Soy Bean Products (Street and Bailey²).

	Soy bean meal.	Soy bean flour.	
	Calculated on water-free basis.	Calculated on water-free basis.	As procured.
	per cent	per cent	per cent
Protein (N \times 6.25).....	36.8-45.5	43.3-45.0	39.9-45.7
Fat.....	14.1-19.0	19.0-22.4	18.2-21.4
N-free extract.....	26.2-32.9	23.5-27.0	22.4-25.8
Fiber.....	4.0- 6.5	2.0- 5.7	1.9- 5.4
Ash.....	5.2- 8.6	4.4- 5.3	4.1- 5.1
Water.....			3.0- 7.8

Street and Bailey² estimated the meal to contain on an average 4.5 per cent of total sugars, 0.5 per cent starch, 3.1 per cent dextrin, 4.9 per cent pentosan, 4.9 per cent galactan, 3.3 per cent cellulose, 1.4 per cent organic acids, and 8.6 per cent waxes, color principles, etc.

Recent studies in nutrition have shown the importance of supplementing our knowledge of the chemical composition of naturally occurring foods by a "physiological" investigation of the availability and suitability of the groups of nutrients which they contain. This has already been emphasized, for example, in the case of corn, wheat, certain legumes, etc.³

² For the leguminous proteins see Osborne, T. B., and Mendel, L. B., *Z. physiol. Chem.*, 1912, lxxx, 21; *J. Biol. Chem.*, 1914, xviii, 1; McCollum, E. V., Simmonds, N., and Pitz, W., *J. Biol. Chem.*, 1917, xxix, 521.

For the maize kernel see Osborne and Mendel, *Carnegie Institution of Washington, Publication No. 156*, pt. ii, 1911; *Z. physiol. Chem.*, 1912, lxxx, 21; *J. Biol. Chem.*, 1912-13, xiii, 233; 1914, xvii, 325; xviii, 1, 95; 1916, xxv, 1; 1916, xxvi, 293; 1917, xxix, 69, 289. Hogan, A. G., *ibid.*, 1916, xxvii, 193; 1917, xxix, 485. McCollum, Simmonds, and Pitz, *ibid.*, 1916-17, xxviii, 153, 483.

For the cotton seed see Osborne and Mendel, *J. Biol. Chem.*, 1917, xxix, 69, 289. Richardson, A. E., and Green, H. S., *ibid.*, 1916, xxv, 307; 1917, xxx, 243; xxxi, 379.

Many other illustrations have been summarized by McCollum, *J. Am. Med. Assn.*, 1917, lxviii, 1379 (this discusses some of the underlying principles involved). See also Mendel, L. B., *ibid.*, 1915, lxiv, 1539.

Our recently published experiments in the study of the use of cotton seed as food,⁴ as well as agricultural practice, show the danger of drawing conclusions as to the nutrient value of natural products on the basis of the customary chemical analysis alone; and they demonstrate the occasional possibility of converting an inappropriate food into a desirable one by a simple expedient,—by the application of heat, in the case of the cotton seed.

In testing the nutritive possibilities of various soy bean products we have employed white rats which were fed according to the plan followed for many years in our earlier investigations. When the question of the suitability of the *protein* alone was involved the other ingredients of the diet were furnished in the form of "protein-free milk," starch, butter fat, and lard. That at least one of the proteins—glycinin⁵—of the soy bean can facilitate growth satisfactorily has already been indicated.⁶ Likewise the suitability of the proteins of the soy bean as supplements to the corn proteins has been demonstrated.⁷

Experiments in which raw soy bean meal⁸ was used as the source of protein in the diet indicated that in the preponderating number of cases the rats made comparatively little growth, despite the addition of the known essential ingredients other than protein. On the basis of our experience with the cotton seed, we attempted to find some method of treating the beans which would render them as suitable for nutrition as the commercial soy bean "flour" which we had used. Extracting the meal with ether failed to increase its acceptability (see Rats 4096, 4094, Chart I, first period). In this respect the soy bean differs from the cotton seed. Heating the soy bean meal in an electric oven at 110°C. for 4 hours likewise failed to cause any considerable improvement

⁴ Osborne and Mendel, *J. Biol. Chem.*, 1917, xxix, 289.

⁵ For the preparation and properties of glycinin see Osborne, T. B., and Campbell, G. F., *J. Am. Chem. Soc.*, 1898, xx, 419. Osborne, T. B., *Ergebn. Physiol.*, 1910, x, 47; and Abderhalden's *Handb. biochem. Arbeitsmethoden*, 1910, ii, 311.

⁶ Osborne and Mendel, *Z. physiol. Chem.*, 1912, lxxx, 324, Curve 20.

⁷ Osborne and Mendel, *J. Biol. Chem.*, 1917, xxix, 69.

⁸ The meal was prepared in the laboratory by grinding the entire bean in a Hobart electric mill. After putting the beans through the mill twice, the meal was sifted through a fine wire sieve and the bits of hull which would not pass through the mesh were thrown away.

in most cases (see Rats 4145, 4144, Chart I). Occasionally, when the animals would eat more freely of the dry-heated meal they grew fairly well (see Rats 4164, 4135, 4142, Chart I). When, on the other hand, the meal was mixed with sufficient distilled water to make a thick mush, heated on the steam bath for 3 hours, and subsequently dried in a current of air at 80-90°, the resulting product, fed as the sole source of protein in an otherwise suitable food, promoted growth at a normal rate (see all rats, Chart I; Rats 4148, 4160, 4162, 4149, Chart II; Rats 4156, 4137, 4147, 4151, 4338, 4343, 4340, 4339, 4341, 4342, Chart III; Rats 3925, 4181, 4184, 4199, Chart IV). Similar results were obtained when commercial soy cake meal⁹ was used as the source of protein (see Rats 4196, 4104, 4118, 4152, 4110, 4200, 4031, 4141, 4035, 4039, 4335, 4336, 4333, 4337, Chart II; Rats 4076, 4071, 4080, 4070, 4330, 4332, 4329, 4328, 4331, Chart III; Rats 4077, 4083, 4081, 4072, Chart IV). We assume that this soy cake meal was prepared from residues obtained in the extraction of oil; and presumably heat was employed to facilitate this process as it is in the case of other oil-rich seeds, *e.g.*, the cotton seed. It is stated that soy oil is produced at times in the cotton seed oil mills.

The question then arose as to the cause of the variations in the apparent nutritive value of these different preparations. Evidently there is nothing toxic in the raw meal, for none of the rats which ate it died. The failure to grow was seemingly associated with a failure to eat the meal readily; for the few rats which ate liberal amounts of the food made satisfactory growth (see Rat 3925, Chart IV). It is probable, therefore, that cooking the meal made it somewhat more palatable than simply heating it, thereby inducing the rats to consume more of the food, with a resulting gain in weight. Cooking the meal changes its flavor very decidedly, giving it a taste similar to that of peanuts; whereas both the raw and the dry-heated meals have a disagreeable raw flavor.

Incidentally we have studied growth upon diets prepared with soy bean meal sold for human consumption. The results obtained with the four brands investigated differed. With two

⁹ The soy cake meal used was obtained from the Cerec Company, Tappan, N. Y.

preparations good growth was accomplished. With the other two the animals grew only slowly, as in our experiments with dry-heated soy bean meal foods; but when the commercial products were cooked, good growth was obtained in accord with the experience with our own cooked soy bean meal. It seems likely that the different results obtained with the commercial soy bean flours are due to unlike methods of heating in their preparation.

A study of the alimentary utilization of the different products was also made to see if that factor would throw any light on the differences between them. Although there is no marked variation between the utilization of the nitrogen of the raw meal and the cooked, such as was previously found to be the case with phaseolin, the protein of the kidney bean, nevertheless the nitrogen of the commercial soy cake and the cooked meal was utilized somewhat better than was the case with the raw and dry-heated meals. It is conceivable that the process employed in manufacturing the soy cake in the one case, and the method of cooking used in the other, tended to get the meal into a more finely divided condition than could be done by merely grinding the beans, and thus rendered the protein more accessible to the action of the digestive enzymes.

*Nitrogen Utilization.**

	<i>per cent</i>
<i>Raw</i> soy bean meal.....	78.0 (76.1-79.9)
<i>Heated</i> soy bean meal.....	72.8 (70.8-75.8)
<i>Cooked</i> " " "	82.0 (80.3-84.3)
Commercial soy cake meal.....	82.2 (81.1-83.6)

* These figures represent the average results for three or four rats taken over a period of 3 weeks.

These utilization figures correspond very well with those obtained by Mendel and Fine¹⁰ for both men and dogs. With the cooked beans they found that men utilized about 85 per cent of the nitrogen and dogs 75 to 88 per cent. Oshima¹¹ states that the digestibility of the protein of various Japanese soy bean products is 79 to 93 per cent.

Having demonstrated that the *proteins* of the soy bean, unlike

¹⁰ Mendel, L. B., and Finé, M. S., *J. Biol. Chem.*, 1911-12, x, 433.

¹¹ Oshima, K., *U. S. Dept. Agric., Office of Experiment Stations, Bull.* 159, 1905

those of the other leguminous seeds thus far investigated, *are adequate for promoting normal growth*, it was important to determine to what extent the soy bean is capable of furnishing the essential vitamins and salts of the diet. On a ration consisting of soy cake meal or of cooked soy bean meal together with an artificial salt mixture,¹² starch, butter fat, and lard, rats have completed their normal growth, showing that *the soy bean contains an adequate amount of the "water-soluble vitamin."* When lard entirely replaced the butter fat rats have grown normally for over 200 days without showing any symptoms of a nutritive decline. This is decidedly longer than the majority of our rats have grown on a diet without the addition of fat-soluble vitamin derived from special sources like butter fat. It seems, therefore, that the soy bean contains some of the essential fat-soluble vitamin. In harmony with this we have never observed any symptoms of eye disease which commonly are manifested by rats kept for some time without a suitable supply of the "fat-soluble vitamin."¹³ The only admixture besides lard to the soy bean meals in the diet of the animals represented on Chart IV was starch and inorganic salts. Other illustrations of rats which have grown on the soy bean meal-lard diets (with the addition of natural "protein-free milk") are represented on Chart II, Rats 4152, 4110, 4141, 4039. It should be noted, however, that in general the growth of the animals on the soy bean-lard

¹² The composition of the salt mixture used is as follows.

	gm.		gm.
CaCO ₃	134.8	Citric acid + H ₂ O....	111.1
MgCO ₃	24.2	Fe citrate 1 $\frac{1}{2}$ H ₂ O.....	6.34
Na ₂ CO ₃	34.2	KI.....	0.020
K ₂ CO ₃	141.3	MnSO ₄	0.079
H ₃ PO ₄	103.2	NaF.....	0.248
HCl.....	53.4	K ₂ Al ₂ (SO ₄) ₃	0.0245
H ₂ SO ₄	9.2		

The chemicals used were analyzed and allowance was made for moisture, etc. The acids were mixed and the carbonates and ferric citrate added to them. The traces of KI, MnSO₄, NaF, and K₂Al₂(SO₄)₃ were added as solutions of known concentrations. The final resulting mixture was evaporated to dryness in a current of air at 90-100°C. and ground to a fine powder.

¹³ Osborne and Mendel, *J. Biol. Chem.*, 1913-14, xvi, 423; 1914, xvii, 401; 1915, xx, 379.

diets has not been as satisfactory as in those cases in which butter fat was furnished in the ration (Chart III). Furthermore it appears as if the animals receiving along with the lard only the soy cake meal (from which much oil has been expressed) as the source of "fat-soluble vitamines" grew less satisfactorily than the rats on the whole soy bean meal-lard diets. Whether this is merely a coincidence or whether some of the "fat-soluble vitamines" has been expressed with the oil, we are not yet able to decide.

The majority of our rats kept on a diet consisting of cooked soy bean meal or of soy cake meal with the addition of starch, butter fat, and lard, but without added mineral ingredients, have failed to make any appreciable gain in weight. The addition to the food of 5 per cent of an artificial salt mixture, however, induced an immediate resumption of growth, thus proving that from the standpoint of a perfect mixture of nutrients *the soy bean is deficient in its mineral constituents* (see Chart V). An analysis of the ash of the meal and the soy cake used in our experiments gave the following results:

Per cent of the air-dry material.	Soy cake meal.	Soy bean meal.
	<i>per cent</i>	<i>per cent</i>
Total ash.....	5.18	5.43
Calcium.....	0.34	0.18
Magnesium.....	0.27	0.30
Potassium.....	1.86	2.06
Sodium.....	0.17	0.14
Chlorine.....	0.01	0.005
Phosphorus.....	0.60	0.82

These figures show that the soy bean is relatively poor in calcium and chlorine, and suggest these as limiting factors. The addition of a small amount of calcium carbonate to a food in which the soy bean was the sole source of mineral constituents tended to stimulate growth to some extent, though not so efficiently as the addition of the more complex salt mixture mentioned above. During these experiments distilled water only was supplied to the rats.

On diets containing either the soy bean meal or the commercial

soy cake meal, together with fats and "protein-free milk" or our "artificial" salt mixture, several broods of vigorous young have been produced, and these young have grown normally on diets the same as those on which their parents were raised. This is a further demonstration of the nutritive efficiency of this legume, in striking contrast with the adverse results obtained with kidney beans and garden peas.

So far as we are aware the soy bean is the only seed hitherto investigated, with the possible exception of flax and millet,¹⁴ which contains both the water-soluble and the fat-soluble unidentified dietary essentials or vitamins. This fact, taken with the high physiological value of the protein, lends a unique significance to the use of the soy bean as food.¹⁵

¹⁴ McCollum, E. V., and Pitz, W., *J. Biol. Chem.*, 1917, xxxi, 229.

¹⁵ Since this paper went to press an article by Daniels, A. L., and Nichols, N. B., The nutritive value of the soy bean, appeared in *J. Biol. Chem.*, 1917, xxxii, 91.

CHARTS.

CHART I. Showing the growth of rats on diets containing *soy bean meal as the source of protein*. The favorable effect of cooking the meal (period——) in contrast with dry-heated meal (period — . — . —) and particularly raw meal (period ———) is shown. In the case of Rats 4096 and 4094 the raw meal was extracted with ether (in the first period ———) without improving the acceptability of the ration, in contrast with what happens in the case of the cotton seed meal (see Osborne and Mendel, *J. Biol. Chem.*, 1917, xxix, 289).

The composition of the diets is given below.

	Period ———						Period — . — . —		Period ——		
	Rats.						Rats.		Rats.		
	4094	4095	3926	3929	4135	4138	4135	4142	4094	3926	4135
	4096	4097	3928	3931	4139	4144	4145	4144	4095	3928	4139
					4142			4164	4096	3929	4145
					4145				4097	3931	
										4138	
										4142	
										4144	
										4164	

Percent.

Raw soy bean meal . . .		53	53	53	53	53					
“ “ “ “											
extracted with ether. 38											
Soy bean meal heated											
dry at 110°C.							50	50			
Cooked soy bean meal.									50	50	50
“Natural protein-free											
milk”	28	22							25		
“Artificial protein-free											
milk”*			22	22							
Salt mixture.					5	5	5	5		5	5
Starch.	2				17	17	20	20		20	20
Butter fat.	18	18	18			18		18	18	18	
Lard.	14	7	7	25	25	7	25	7	7	7	25

* The “artificial protein-free milk” employed is described in *J. Biol. Chem.*, 1913, xv, 317. The one marked “IV” was used.

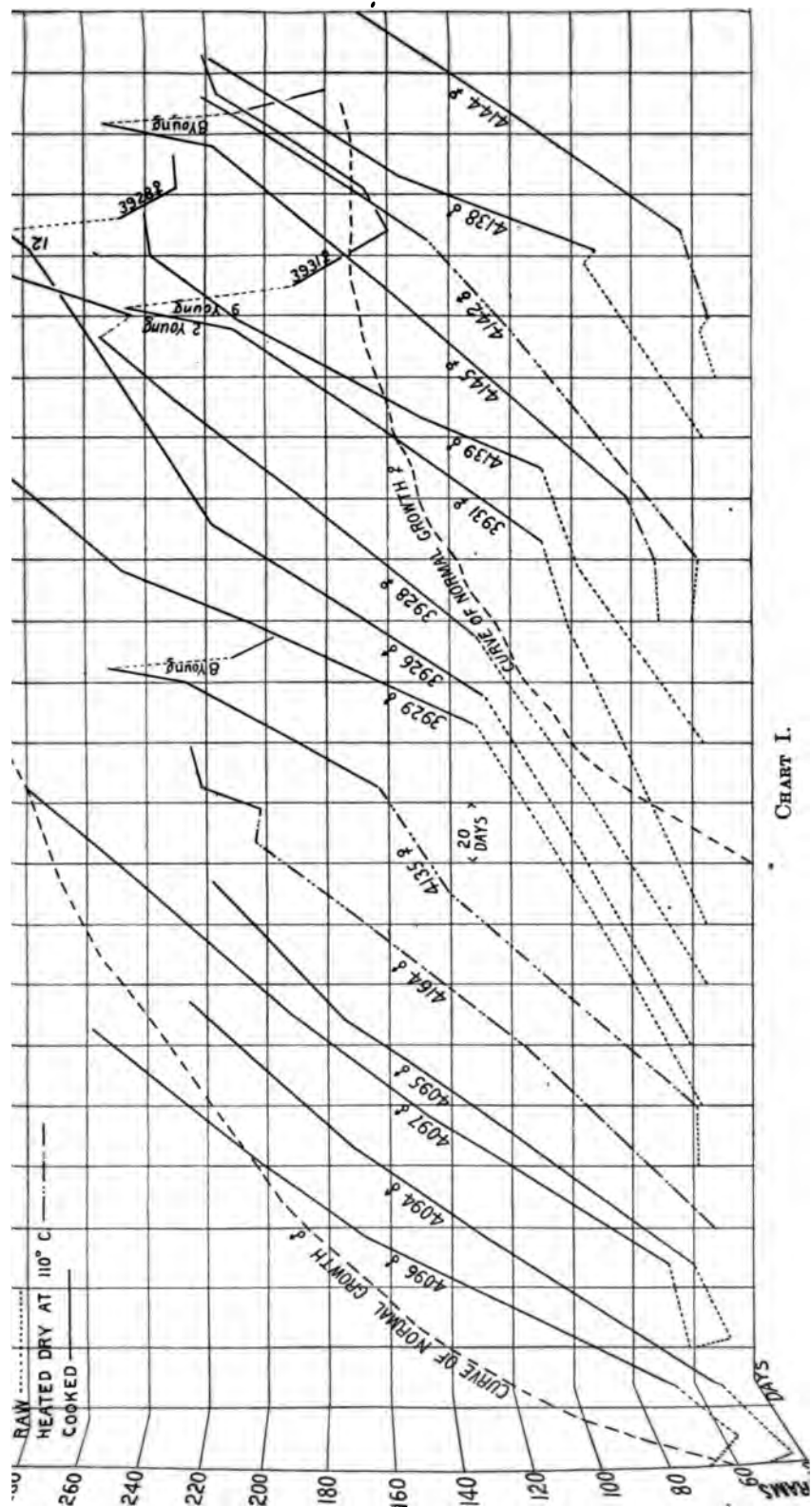


CHART I.

CHART II. Showing excellent growth of rats from an early period on diets containing soy bean meal (cooked) or soy cake meal (which has been heated during the commercial removal of the oil) as the source of protein in the diet. Several of the females were mated and gave birth to litters of normal young which thrive on the same diet. Curves of growth for some of these are reproduced at the right-hand side of the chart.

The composition of the diets is given below.

	Rats		Rats		Rats	
	4148 4160	4149 4162	4031 4104 4196 4333 4336	4035 4118 4200 4335 4337	4035 4110 4152	4039 4141
	<i>per cent</i>		<i>per cent</i>		<i>per cent</i>	
Soy bean meal (cooked).....	50					
“ cake “			37.5		37.5	
“Protein-free milk”.....	25		28.0		28.0	
Starch.....			10.5		10.5	
Butter fat.....	18		18.0			
Lard.....	7		6.0		24.0	

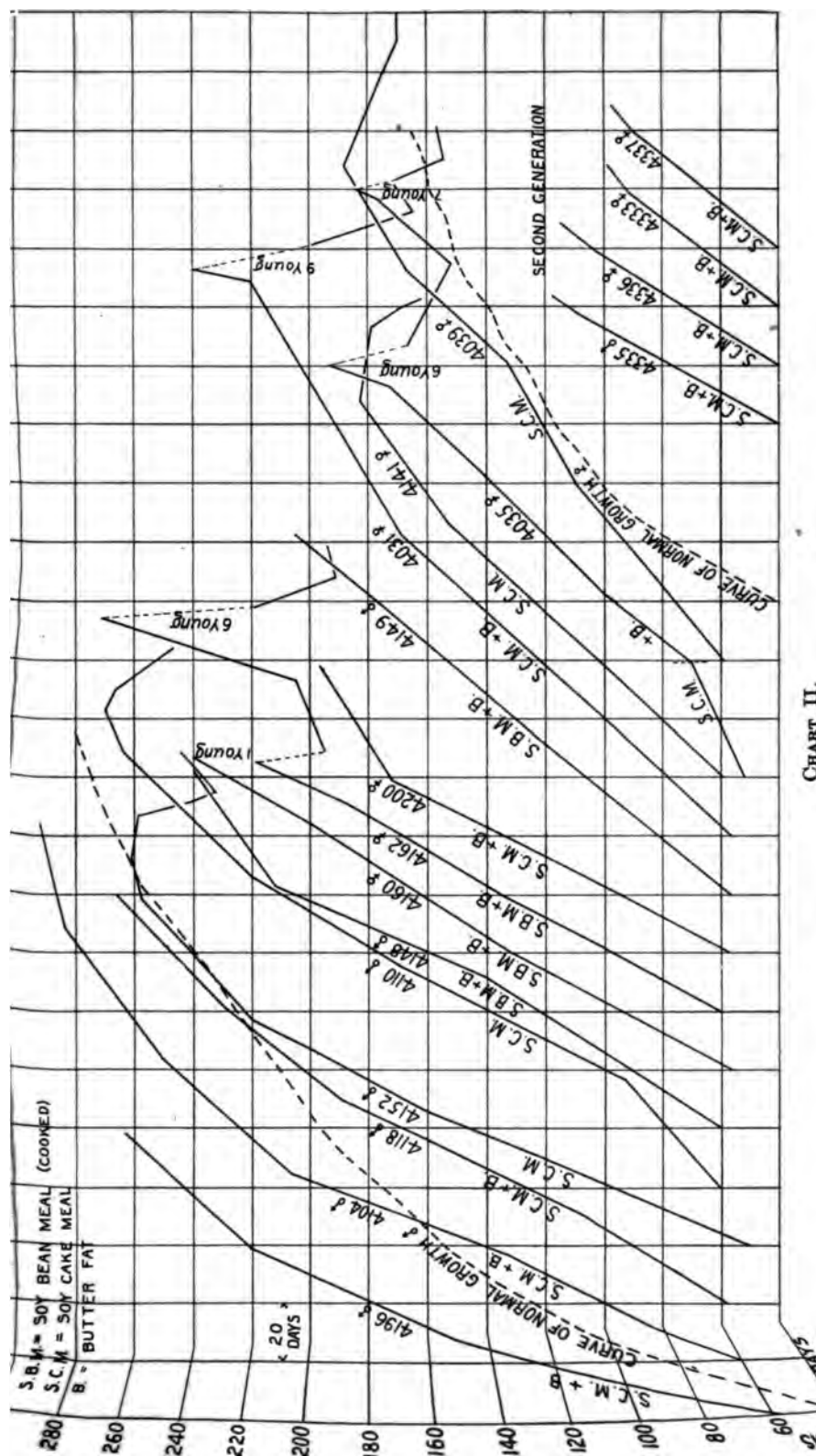


CHART III. Showing growth and reproduction on diets containing cooked soy bean meal or soy cake meal, which has been heated during the commercial removal of the oil, as the sole source of protein and "water-soluble" vitamins in the diet. Additional "fat-soluble" vitamins were given in the form of butter fat in contrast to the experiments shown in Chart IV which had no butter fat in the diet. Several of the females were mated and gave birth to litters of normal young which thrived on the same diet. Curves of growth for some of these are reproduced at the right-hand side of the chart.

The composition of the diets is given below.

	Rats			Rats		
	4137 4156 4340 4343	4147 4338 4341	4151 4339 4342	4070 4080 4330	4071 4328 4331	4076 4329 4332
	<i>per cent</i>			<i>per cent</i>		
Soy bean meal (cooked).....	50					
" cake "				37.5		
Salt mixture.....	5			4.5		
Starch.....	20			38.0		
Butter fat.....	18			15.0		
Lard.....	7			5.0		

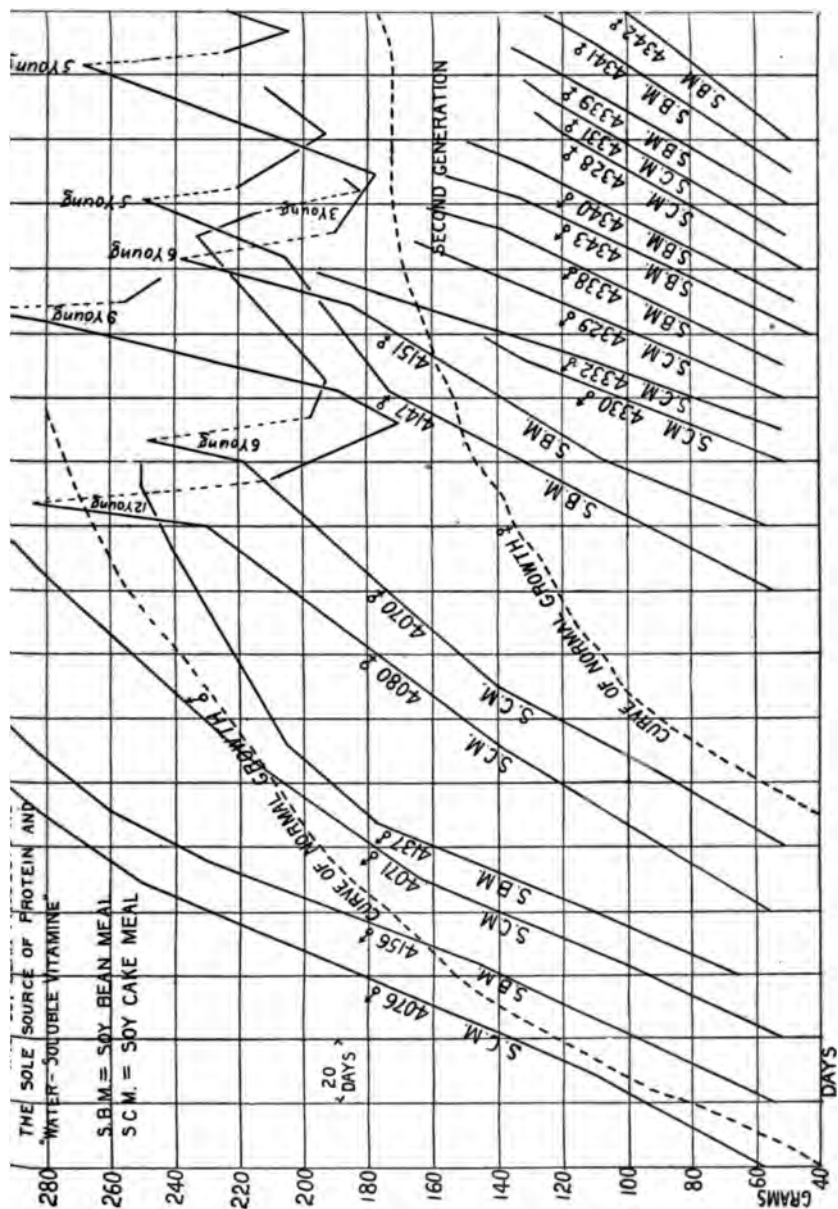


CHART IV. Showing growth on diets containing soy bean meal or soy cake meal as the sole source of protein and "vitamines" in the diet. These experiments show that suitably prepared soy bean meals, supplemented by appropriate inorganic salts, furnish practically all of the essential nutrients. The experience with Rat 3925, which grew to considerable size on a diet containing the soy bean meal in its raw form shows that in contrast with cotton seed meal the product is not toxic, and can be utilized as a nutrient, provided that it is eaten in suitable amounts. The ultimate failure of this rat to grow further on this raw meal diet was reversed when the meal was cooked. None of these animals received fat in any other form than that contained in the soy bean and in lard. The bean must therefore contain some "fat-soluble vitamine." One gains the impression that the animals receiving the whole bean meal (including all of its oil) grew somewhat more satisfactorily than those receiving the soy cake meal (with much oil expressed) as the source of "fat-soluble vitamine." Whether the quantity of the "fat-soluble vitamine" thus furnished is sufficient to permit all animals to complete their growth without additions from other sources remains to be determined. At any rate when butter fat is added somewhat better growth is uniformly obtained over longer periods (see Chart III).

The composition of the diets is given below.

	Period ----	Period ———		Rats	
	Rat 3925	Rats		Rats	
		3925	4181	4672	4677
		4184	4199	4681	4683
	<i>per cent</i>	<i>per cent</i>		<i>per cent</i>	
Soy bean meal (raw).....	53	50		37.5	
“ “ “ (cooked).....					
“ cake “					
Salt mixture.....	5	5		4.5	
Starch.....	17	20		38.0	
Lard.....	25	25		20.0	

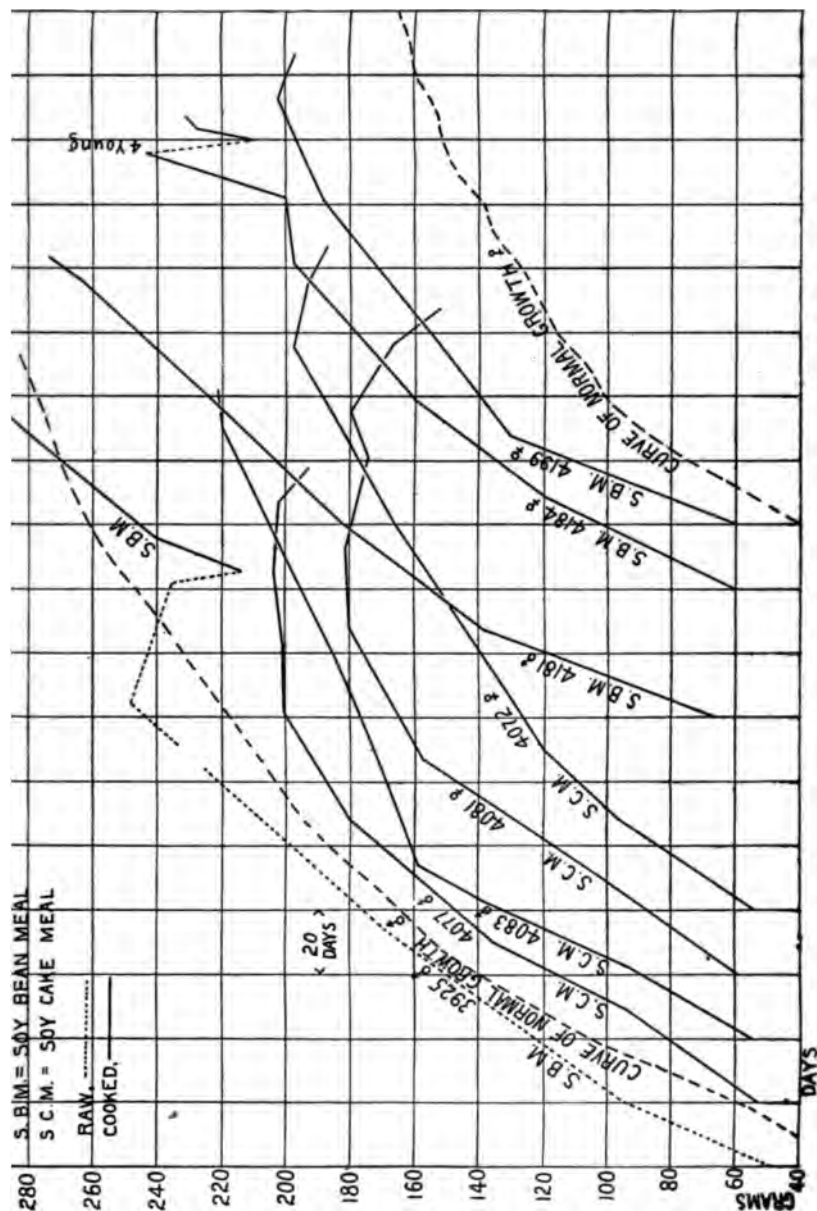


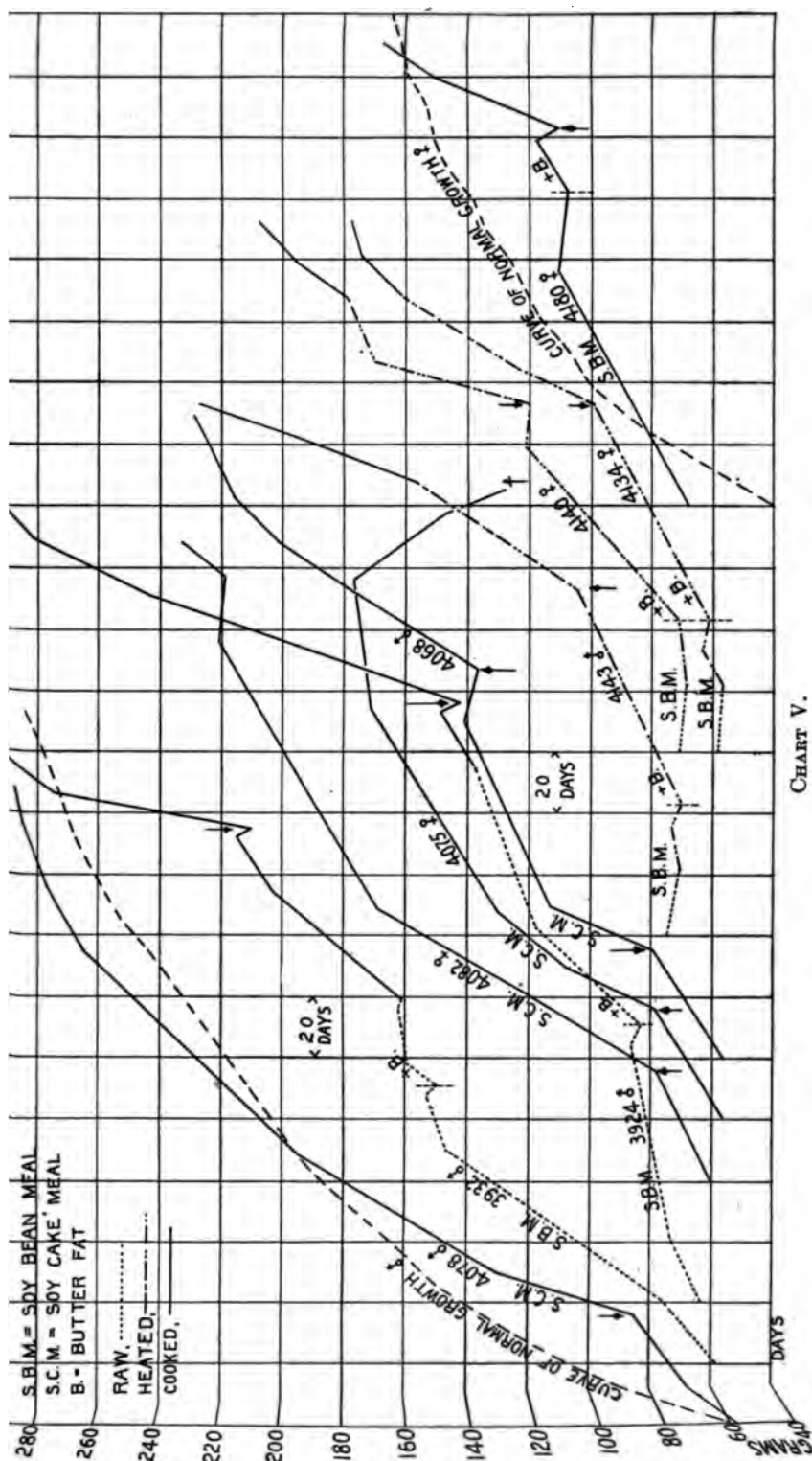
Chart IV

CHART V. Showing the prompt improvement in growth when a mixture of inorganic salts (see page 375) was added to the rations consisting of soy bean or soy cake meal, starch, and fat. The beginning of the period during which the added salts were fed is indicated by an arrow.

The periods during which butter fat was fed are indicated by B; otherwise lard was used as the fat. The contrast between the cooked and uncooked meal is shown in several instances. It is sometimes missing and never so conspicuous as on the previous charts, owing to the fact that the lack of adequate inorganic salts forms a limiting factor in growth which no amount of cooking of the meal can remedy.

The diets had essentially the following composition:

	Without added salts.				With added salts.			
	Rate		Rate		Rate		Rate	
	3924	3932	4068	4075	3924	3932	4068	4075
	4134	4140	4078	4082	4134	4140	4078	4082
	4143	4180			4143	4180		
	<i>per cent</i>		<i>per cent</i>		<i>per cent</i>		<i>per cent</i>	
Soy bean meal.....	50-53				50			
“ cake “			37.5				37.5	
Starch.....	24-27		42.5		20		38.0	
Fats.....	23		20.0		25		20.0	
Salt mixture.....					5		4.5	



STUDIES OF THE GASTRIC RESIDUUM. II.

TOTAL PHOSPHORUS.

By CHESTER C. FOWLER.

*(From the Laboratory of Physiological Chemistry, Department of Chemistry,
Iowa State College, Ames.)*

(Received for publication, October 22, 1917.)

INTRODUCTION.

Support of a modification of Maly's (1) hypothesis concerning the formation of the hydrochloric acid of the gastric juice has recently been presented by Bergeim (2). He has suggested that the acid is produced through the decomposition of sodium chloride by acid calcium phosphate, which is obtained from cell nuclei by the action of phosphonuclease (present in nearly all tissues). Bergeim assumes that the gastric juice might be expected to contain small amounts of acid calcium phosphate, and that these amounts might be roughly proportional to the acidity. Of both of these assumptions he has claimed to have supporting data.

It seemed to the writer to be desirable in this connection to make a complete study of phosphorus and its partition in the gastric residuum and to this end this study of total phosphorus has been undertaken. As far as we have been able to ascertain, no systematic investigations of this nature have been pursued, nor have we been able to find such data on the pure gastric juice. In this laboratory (3) the total phosphorus content of a composite residuum, obtained from 70 men, has been determined and was found to be 12.16 mg. (P_2O_5) per 100 cc. of the original residuum.

Methods.

52 samples of gastric residuums were procured from apparently normal women. These residuums were obtained by means of the Rehfuß gastroduodenal tube according to methods described elsewhere (4). The volumes of the samples were immediately

determined and the physical characteristics noted. The determinations, total acid, free acid, and pepsin were then made on each sample and the remaining portion was transferred to a Kjeldahl flask and its total phosphorus content determined. Portions were used for trypsin determinations in another investigation.

The methods for total acid, free acid, and pepsin were those used by Rehfuss, Bergeim, and Hawk (5), except that pepsin was incubated 14 hours instead of 12. Total phosphorus was determined by destroying the organic matter by digestion with a mixture of sulfuric and nitric acids. The phosphorus was then precipitated as the phosphomolybdate, and finally the molybdic acid was titrated with standard alkali, according to the method first suggested by Pemberton (6).

DATA AND DISCUSSION.

TABLE I.

Analytical Results on Gastric Residuums.

Sample No.	Volume.	P ₂ O ₅ per 100 cc. original.	Total acid, 0.1 N acid per 100 cc. original.	Free acid, 0.1 N acid per 100 cc. original.	Pepsin, square root of the concentration.
	cc.	mg.	cc.	cc.	
22	49	13.20	8.7	Trace.	3.5
24	66	13.82	19.5	3.9	4.0
25	65	12.16	43.7	15.1	4.8
28	35	17.15	11.0	0.0	3.0
31	40	11.26	25.3	9.0	3.5
32	32	6.60	24.1	7.6	4.3
33	79	8.52	48.9	19.4	3.4
34	39	6.48	35.3	11.4	3.0
35	41	14.35	19.2	5.3	3.5
67	61	26.58	9.9	3.0	3.0
69	64	15.62	36.0	23.4	2.1
70	71	12.99	75.0	48.0	4.0
73	53	14.54	4.0	Trace.	2.4
74	86	20.24	43.3	33.0	2.5
76	35	15.08	38.5	19.5	4.1
77	46	9.31	56.0	41.0	4.1
78	88	8.20	59.8	58.0	4.0
80	108	10.17	59.0	42.2	3.8
81	55	14.30	26.6	10.0	4.9
82	23	21.11	45.5	30.0	6.7
83	23	30.03	7.8	Trace.	4.4
84	19	11.23	38.7	22.1	5.1
85	11	24.20	11.1	0.5	1.3
86	30	17.37	34.9	22.3	2.0
87	23	19.27	34.5	17.7	2.5
88	33	18.28	86.0	74.5	3.0
89	28	17.57	12.6	1.4	2.3
90	96	15.37	48.9	34.6	3.0
91	31	17.17	12.0	2.0	2.0
92	31	20.57	32.0	18.0	4.0
93	21	24.96	14.0	3.0	2.7
94	131	21.75	26.1	8.2	3.0
95	86	15.21	43.9	33.3	5.0
96	49	16.22	51.5	35.0	4.5
97	38	14.28	33.0	18.5	5.3
98	36	9.44	5.0	2.5	2.0
99	34	13.73	34.5	13.5	2.4
100	38	12.85	12.7	3.0	4.3
101	28	16.43	26.3	11.0	3.1
102	31	17.34	13.5	3.2	3.3

TABLE I—*Concluded.*

Sample No.	Volume.	P ₂ O ₅ , per 100 cc. original.	Total acid, 0.1 N acid per 100 cc. original.	Free acid, 0.1 N acid per 100 cc. original.	Pepsin, square root of the concentration.
	cc.	mg.	cc.	cc.	
105	50	26.87	25.4	10.0	3.8
106	58	15.31	7.4	1.0	0.0
109	56	17.51	24.2	8.1	3.5
111	56	15.97	43.0	26.3	3.9
112	54	17.56	27.0	10.7	4.3
113	79	11.78	35.3	25.5	3.5
114	64	13.36	31.6	15.0	3.4
115	30	16.08	58.8	39.5	5.3
116	74	13.41	26.0	11.5	4.5
117	50	11.24	55.0	50.5	2.8
118	61	12.87	12.0	11.4	2.0
119	26	17.40	36.5	26.9	1.0

Several of the subjects of this investigation presented themselves twice for examination. These are represented in Table I as follows: Subject E. B., Samples 99 and 118; Subject M. N., Samples 35 and 89; Subject M. B., Samples 33 and 78; Subject E. M., Samples 24 and 109; Subject Z. Z., Samples 25 and 28; Subject J. R., Samples 97 and 116; Subject M. J., Samples 67 and 94; Subject L. S., Samples 98 and 106; and Subject M. E., Samples 34 and 91. Of these nine subjects it will be seen from the table that the first seven show a remarkably constant value for the P₂O₅ content of their respective residuums notwithstanding the fact that those residuums were collected at widely different times. The other two do not show this constancy.

SUMMARY.

1. Total phosphorus seems to bear no relation to total acid, free acid, pepsin, or volume.
2. The minimum P₂O₅ content was 6.48 mg. per 100 cc. and the maximum was 30.03 mg.
3. About 58 per cent of the samples fell within the range P₂O₅ equivalent to 12 to 18 mg., while about 21 per cent lay above and 21 per cent below these values.
4. A tendency toward a constant P₂O₅ content was shown in individuals who were examined more than once.

5. The average P_2O_5 content was 15.66 mg. In a previous investigation made upon a composite residuum sample, obtained from 70 men, a value of 12.16 mg. of P_2O_5 per 100 cc. of residuum was obtained.

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STUDIES ON ENZYME ACTION.

XVI. THE FORMATION OF ESTER-HYDROLYZING SUBSTANCES BY THE ACTION OF ALKALI ON PROTEINS.

By FLORENCE HULTON-FRANKEL.

(From the Harriman Research Laboratory, The Roosevelt Hospital,
New York.)

(Received for publication, August 11, 1917.)

An extended series of studies on enzyme action that is being carried on in this laboratory has brought to light the fact that some proteins when treated with alkali acquire ester-hydrolyzing properties (Falk, 1916, 1917). The present investigation was undertaken to determine whether the activity of these ester-hydrolyzing substances follows the general laws of enzyme action, and to what extent they are specific in their action.

The proteins used in this investigation were casein (Kahlbaum's according to Hammersten), gelatin, and dried egg albumen, the latter two being commercial products. The esters used were of a high order of purity and were in most cases redistilled after drying over sodium carbonate.

Influence of Concentration of Alkali Used and Duration of Action.

Experiments with Casein.—2 gm. portions of the protein were treated with 25 cc. of alkali of varying concentration (5 to 0.1 N), thoroughly shaken, and toluene was added and allowed to stand for 24 hours. The mixtures were then turbid yellowish brown solutions. They were either neutralized with N HCl after dilution or dialyzed in collodion bags against running water. When the solutions were directly neutralized about 500 cc. of water were first added and then enough N HCl was added to bring the H ion concentration to 10^{-8} N, the indicator method (Clark and Lubs, 1917) being used to define this point. When the alkali was removed by dialysis very little acid was necessary

to adjust the solution to the H ion concentration desired. The final volume of the solution in all cases was 500 cc. A small amount of the protein solution was used in adjusting the H ion concentration before the final dilution was made, but the amount so used was negligible. To test the activity of the preparations 50 cc. of the solution were incubated for 24 hours with 1 cc. of ethyl butyrate and 0.5 cc. of glyceryl triacetate respectively, these amounts of ester providing a large excess of substrate. All experiments were run in duplicate and suitable controls for ester and casein preparations carried out. Toluene was used as a preservative in all cases where a preservative was used. It was found by bacteriological controls that no bacterial action took place in any of the alkaline protein solutions, even when no preservative was used. The solutions after incubation were titrated with 0.1 N alkali using phenolphthalein as an indicator. To the neutralized solutions 10 cc. of neutralized 40 per cent formaldehyde were added and the solution was again titrated to a faint pink with phenolphthalein. All results as given are averages of closely agreeing duplicates, after all corrections for blanks were made.

In the following tables are included experiments made to determine the influence of varying the time during which the casein stood in contact with the alkali, all results being expressed in cc. of 0.1 N alkali. The table shows clearly that 3 N alkali gives a product of maximum activity under the given conditions and thereafter in other experiments this concentration of alkali was used. Another fact that is clearly brought out is that the lipolytic activity of the preparations does not bear any simple numerical relation to the formol titration although the action and the formol titration vary in the same direction.

The figures in those experiments in which the alkaline casein solution was dialyzed showed that to a great extent the lipolytically active substance is dialyzable or destroyed in the process of dialysis. However, it does not seem likely that the lipolytic action is due entirely to the simple hydrolysis products of casein, as these would probably have dialyzed out completely in less than 48 hours, while there is slight lipolytic activity in the bag contents even after 48 hours' dialysis when more dilute alkali was used.

The ester-hydrolyzing action of the casein did not seem to be affected by the length of time that the alkali stood in contact with it. The formol titration becomes greater with longer time of standing, while the activity is as great at times in the case where the solution was neutralized directly as when the solution stood for 72 hours.

Experiments with Gelatin.—In the experiments where gelatin was used as the protein, the conditions were almost the same as in the experiments on casein. In the case of this protein the concentrations of alkali used were more limited in range since with dilute alkali the gelatin swelled but did not go into solution. The results for the direct titrations in the experiments to test the activity of the product are not very reliable since it was very difficult to define the end-point. The activity of the preparations is calculated entirely from the total titrations. No experiments in which the gelatin solutions were freed from alkali by dialysis are included in the table, for it was found that when gelatin was allowed to stand with alkali for 24 hours and then dialyzed against running water for 6 hours the bag contents were no longer active as ester-hydrolyzing catalysts.

Only a few experiments were carried out with concentrations of alkali other than 3 N, as this strength of alkali gave a product whose activity was as great as when higher concentrations were employed. In determining the influence of the time of standing 3 N alkali was used throughout. In the case of gelatin in contrast to casein the activity of the product increased with time of standing. The apparent discrepancies are possibly due to the influence of temperature as the room temperature varied quite considerably in the different experiments. This will be discussed in more detail below. While not very accurate, owing to the difficulty of defining the end-point in the direct titration, the formol titrations in this series of experiments indicate that cleavage of the gelatin with alkali is more rapid and extensive than with casein.

Experiments with Egg Albumen.—The conditions in these experiments were the same as under casein and gelatin. Alkali in strengths of 5 N to 0.5 N was used. 3 N alkali was used in the experiments in which the effect of time was studied. As only small activities were noted after 24 hours' contact with the alkali no experiments of shorter duration were carried out.

TABLE I.
Hydrolytic Action of Protein Treated with Alkali.

Concentration of NaOH.	Time of standing.	Formol titration. 0.1 N alkali.	Actions.			
			Ethyl butyrate.		Triacetin.	
			0.1 N alkali.		0.1 N alkali.	
Casein.						
	hrs.	cc.	cc.	pH	cc.	pH
5 N	24 neutralized.	1.70	0.28	7	0.60	6
	72 dialyzed.	0.05	-0.13	8	-0.10	7
	72 neutralized.	1.92	0.30	8	0.83	6
3 N	0 "	0.47	0.07	7	0.33	6
	48 dialyzed.	0.23	0.00	8	0.00	7
	72 "	0.05	0.09	8	0.00	7
	72 neutralized.	2.35	0.18	8	0.86	6
	24 "	1.90	0.16	7	0.81	6
	24 dialyzed.	0.50	0.05	7	0.25	6
2 N	24 neutralized.	1.55	-0.02	7	0.47	6
	48 dialyzed.	0.05	0.02	6	0.11	6
	72 "	0.05	-0.05	8	0.05	7
	72 neutralized.	2.00	0.35	8	0.78	7
1 N	0 "	0.50	0.20	7	0.66	6
	24 "	1.05	0.15	8	0.61	7
	48 dialyzed.	0.17	0.22	7	0.20	6
	72 "	0.40	0.15	7	0.38	6
0.5 N	0 neutralized.	0.65	0.10	6	0.43	6
	24 "	1.00	0.20	7	0.51	6
	48 dialyzed.	0.42	0.05	7	0.30	6
	72 "	0.40	0.08	7	0.28	6
	72 neutralized.	1.40	0.40	7	0.70	7
0.1 N	24 "	0.95	0.20	7	0.30	6
	48 dialyzed.	0.65	0.10	7	0.33	6
	72 "	0.37	0.07	7	0.15	6
	72 neutralized.	0.65	0.05	7	0.35	6
Gelatin.						
5 N	24 neutralized.		0.30	8	0.75	7
3 N	24 "		0.10	8	0.80	7
	48 "		0.35	8	1.30	7
	72 "		0.40	7	0.70	7
	6 "		-0.05		0.50	
2 N	24 "		0.00	8	0.75	7

TABLE I.—*Concluded.*

Concentration of NaOH.	Time of standing.	Formol titration. 0.1 N alkali.	Actions.			
			Ethyl butyrate.		Triacetin.	
			0.1 N alkali.		0.1 N alkali.	
Egg albumen.						
	hrs.	cc.	cc.	pH	cc.	pH
5 N	24 neutralized.	1.62	0.17	8	0.45	6
3 N	24 "	1.55	0.25	7	0.47	6
	48 "	1.40	0.23		0.75	6
	72 "	2.00	0.43	8	0.88	6
2 N	24 "	1.45	0.30	8	0.40	6
1 N	24 "	0.80	0.10	7	0.20	6
0.5 N	24 "	0.80	0.05	6	0.20	6

Egg albumen as a whole gave products of smaller activity than casein or gelatin. The activity of the material as with casein was not very markedly influenced by the time of standing with alkali.

For purposes of comparison a series of experiments was carried out in which a number of proteins were treated with 3 N alkali under the same conditions as obtained in the experiments described above. The proteins were allowed to stand in contact with the alkali at room temperature for 24 hours. In all cases solution of the protein (2 gm.) in the alkali (25 cc.) was complete. The solutions were diluted and treated as in the case of casein, egg albumen, and gelatin. The actions as before are expressed in cc. of 0.1 N alkali required to neutralize the acid produced by the hydrolysis of the esters, suitable corrections having been introduced for all blanks.

It will be observed from Table II that with all the proteins tried lipolytic activity may be induced by treatment of the substances with alkali. The activity of the various preparations is uniformly greater on triacetin than on ethyl butyrate. The comparatively high actions on ethyl butyrate noted in the case of zein and lactalbumin are striking, since with most of the ester-hydrolyzing substances studied the action on this ester is comparatively low.

TABLE II.
Hydrolytic Action of Various Proteins after Treatment with Alkali.

Protein.	Formol titration. 0.1 N alkali.	Actions.	
		Ethyl butyrate. 0.1 N alkali.	Triacetin. 0.1 N alkali.
	cc.	cc.	cc.
Casein.....	1.90	0.16	0.81
Gelatin.....	—	0.10	0.80
Egg albumen.....	1.55	0.25	0.47
Castor bean globulin.....	2.50	0.15	1.12
Zein.....	2.20	0.53	0.80
Phaseolin.....	2.70	0.28	0.88
Edestin.....	2.75	0.10	0.70
Ovitellin.....	1.95	0.15	0.63
Egg globulin.....	2.10	0.15	0.92
Pseudoglobulin.....	1.90	0.00	1.00
Euglobulin.....	2.00	0.30	0.75
Soy bean globulin.....	3.20	0.15	0.75
“ “ glutelin.....	3.00	0.10	0.75
Gliadin.....	4.55	0.27	1.10
Lactalbumin.....	2.10	0.47	1.07

The Influence of the Hydrogen Ion Concentration on the Activity of Alkali-Treated Proteins.

In drawing the analogy between the ester-hydrolyzing substances prepared by the treatment of protein with alkali and lipolytic enzymes, it seemed desirable to determine whether or not a definite H ion optimum for the activity of the substances exists. To this end a series of experiments was set up as before.

Table III shows that the lipolytically active substances prepared from casein and gelatin exhibit their activity in alkaline solution, almost no action being noted at 10^{-6} and 10^{-7} N while there is fair action at 10^{-8} N increasing with the alkalinity. In the case of egg albumen the action at 10^{-8} is very small, while a greater activity is exhibited at 10^{-9} N and does not seem to increase at 10^{-10} N.

TABLE III.
Hydrogen Ion Optimum for Ester Hydrolysis.

pH before incuba- tion.	Casein.				Gelatin.				Egg albumen.			
	Ethyl butyrate.		Triacetin.		Ethyl butyrate.		Triacetin.		Ethyl butyrate.		Triacetin.	
	0.1 N alkali.		0.1 N alkali.		0.1 N alkali.		0.1 N alkali.		0.1 N alkali.		0.1 N alkali.	
10-	cc.	pH*	cc.	pH*	cc.	pH*	cc.	pH*	cc.	pH*	cc.	pH*
6	0.22	6	0.36	6	0.05	6	0.10	6	0.05	6	0.15	6
7	0.10	6	0.40	6	0.00	7	0.10	7	0.00	7	0.00	6
8	0.23	7	0.74	6	0.05	8	0.70	7	0.05	7	0.35	6
9	0.28	8	0.95	7	0.20	8	1.40	7	0.13	7	0.83	6
10	1.00	8	1.38	7	0.95	8	2.80	7	0.30	7	0.80	6

* After incubation.

Influence of Temperature of Standing on the Action of Alkali on Protein.

The temperature at which the alkali stood in contact with the protein seemed to exert a much greater influence than did the time of standing.

The difference in temperature at which the alkali acted on the protein seemed to make little difference at the lower temperatures in the case of casein, as that which had stood in the ice box gave as much action as that which had stood at room temperature, giving practically no action in the case of ethyl butyrate, in either case. The action in the case of gelatin was practically nil for both ethyl butyrate and triacetin where the solution stood in the ice box. The action increased in the casein solution with the temperature of standing while in the case of gelatin at 80°C. the gelatin seemed to dry to a hard mass which did not dissolve readily and the solution was not as active as at 37.5°C. where the gelatin did not dry.

If the ester-hydrolyzing properties are due merely to the hydrolysis products of the protein used, then if acid is allowed to act on protein in the same way as alkali, there should be formed an ester-splitting substance. To test the validity of this assumption 2 gm. portions of casein and gelatin were allowed to stand in contact with 25 cc. N HCl for 24 hours. They were then diluted with 500 cc. of distilled water, neutralized with N NaOH, the H

ion concentration was adjusted to 10^{-8} N, and 50 cc. portions were incubated with ethyl butyrate and triacetin. The titrations corrected for all blanks appear in Table IV contrasted with those obtained with N NaOH under the same conditions.

TABLE IV.
Action Obtained with Solutions Prepared by the Action of HCl on Casein and Gelatin.

	Casein.				Gelatin.			
	Ethyl butyrate.		Triacetin.		Ethyl butyrate.		Triacetin.	
	Action.	Formol titration.	Action.	Formol titration.	Action.	Formol titration.	Action.	Formol titration.
	0.1 N alkali.		0.1 N alkali.		0.1 N alkali.		0.1 N alkali.	
	cc.	cc.	cc.	cc.	cc.		cc.	
N HCl.....	0.20	0.60	0.20	0.60	0.05	*	0.35	*
N NaOH.....	0.15	0.50	0.61	0.50	0.00	*	0.75	*

* Formol titrations could not be determined on account of poor end-point of direct titration.

It would seem as if some factor other than mere hydrolysis must be taken into account as causing the formation of the lipolytically active substance, as much greater action was obtained with weaker alkali than was obtained with the N HCl.

Lipolytic Activity of a Papain Digestion Mixture of Casein.

To test further whether or not hydrolysis products were responsible for the action of the alkaline protein solutions, casein was digested with papain and the resulting solution was incubated with ethyl butyrate and triacetin. 5 gm. of casein were placed in a flask with 125 mg. of Merck's papain and 120 cc. of distilled water were added, some toluene was added, and the flask incubated for 1 week. A control was set up at the same time containing 125 mg. of papain in 120 cc. of distilled water with toluene and the whole incubated for 1 week. At the end of the week two-fifths of the contents of the flask, equivalent to 2 gm. of casein, were diluted to 500 cc. and neutralized, and the H ion concentration was adjusted to 10^{-8} N and 50 cc. portions were incubated with

ethyl butyrate and triacetin. The control was treated in the same way. Bacterial cultures were made from both digestion mixtures, giving negative results. The results corrected for all blanks were as follows:

Ethyl butyrate.	Triacetin.	
0.15	0.35	Formol titration, 1.50

The actions were so small as to come within the error of the method, so that it would seem that hydrolysis alone is not the determining factor in producing a lipolytically active solution. The formol titration of the papain solutions acting on the casein was much greater than that of some of the alkaline solutions where marked action was obtained.

The Effect of Boiling on the Lipolytically Active Substance.

The question of inactivation arose in connection with the lipolytically active substance produced when a protein was treated with alkali. Several attempts were made to inactivate the solutions by boiling. 2 gm. portions of the several proteins were treated with 25 cc. of 3 N alkali and allowed to stand 24 hours. The solutions were then diluted to 500 cc. and the H ion concentrations adjusted to 10^{-8} N and 50 cc. portions incubated with ethyl butyrate and triacetin. The results are recorded below in cc. of 0.1 N NaOH, corrected for all blanks.

TABLE V.
Influence of Boiling on Alkaline Solution of Casein, Gelatin, and Egg Albumen.

Time. min.	Casein.				Gelatin.				Egg albumen.			
	Ethyl butyrate.		Triacetin.		Ethyl butyrate.		Triacetin.		Ethyl butyrate.		Triacetin.	
	0.1 N alkali.		0.1 N alkali.		0.1 N alkali.		0.1 N alkali.		0.1 N alkali.		0.1 N alkali.	
	cc.	pH*	cc.	pH*	cc.	pH*	cc.	pH*	cc.	pH*	cc.	pH*
5	0.25	8	0.40	6								
15	0.15	8	0.50	7	0.15	8	0.95	7				
20					0.35	8	1.35	7				
25									0.08	7	0.78	6
60	0.15	7	0.80	6								

* After incubation.

While the formol titrations remained the same as in the control solutions that were not boiled, the activity of the solution in the case of casein seemed to be somewhat less than usual, but it was not possible to destroy the active principle even when the solution was boiled 60 minutes. In the case of the gelatin and egg albumen the action was not at all affected by the boiling. The apparent increase in action in the case where the gelatin was boiled for the longest interval may be partially due to the fact that the alkali stood in contact with the gelatin at a slightly higher temperature in the second instance. It is obvious that the ordinary methods for inactivation of enzymes could not be used here as acid would only neutralize the alkali used in the beginning, and alkali cannot inactivate it as alkali is responsible for the action. Hamlin (1913), Falk and Nelson (1912), and Falk (1917) working with amino-acids, castor bean lipase, dipeptides, and ethyl imidobenzoate, found that the lipolytic activities of different catalysts varied with different esters. For purposes of comparison with their data a series of experiments was carried out in which the activity of proteins treated with alkali on different esters was determined.

6 gm. portions of the proteins were treated with 75 cc. of 3 N NaOH and allowed to stand at room temperature for 24 hours. The solutions were then diluted to 1,500 cc. and the hydrogen ion concentrations adjusted with N HCl. In the case of egg albumen the H ion concentration was 10^{-9} N while with casein and gelatin the solutions were brought to 10^{-8} N. 50 cc. portions of the solutions were then incubated with 0.5 or 1.0 cc. of the various esters, a large excess of substrate being present in all cases. The results are expressed in cc. of 0.1 N alkali required to neutralize the acid formed per 0.01 gm. molecule of ester used. These results were comparable to those recorded by Falk. In the case of glyceryl triacetate a second calculation was made to express the amount of acid formed per 0.01 gm. equivalent of the ester. For purposes of comparison with the results of Falk, the esters are arranged in the descending order of the action of the ester-hydrolyzing substance on them. Glyceryl triacetate is listed twice, the first being for the calculation made per gm. molecule of the substance, while the second refers to that in which one equivalent is used as a basis of calculation.

Order in Which Esters Are Hydrolyzed in the Presence of Catalyts.

Water.	Glycyl-glycine.†	Ethyl imido-benzoate.†	Casein.	Gelatin.	Egg albumen.
Phenyl acetate.	Phenyl acetate. 3.98*	Glyceryl triacetate. 1.77	Glyceryl triacetate. 3.02	Ethyl benzoate. 3.10	Glyceryl triacetate. 2.64
Methyl acetate.	Glyceryl triacetate. 2.84	Phenyl acetate. 1.62	Methyl benzoate. 2.05	Glyceryl triacetate. 2.64	Phenyl acetate. 1.04
Glyceryl triacetate.	Methyl acetate. 2.40	Methyl acetate. 1.45	Glyceryl triacetate. 1.01	Phenyl acetate. 1.43	Glyceryl triacetate. 0.88
Ethyl acetate.	Ethyl acetate. 1.84	Ethyl acetate. 1.34	Methyl acetate. 0.76	Ethyl acetate. 1.20	Ethyl benzoate. 0.71
Olive oil.	Glyceryl triacetate. 0.95	Glyceryl triacetate. 0.59	Ethyl acetate. 0.75	Methyl acetate. 1.12	Methyl acetate. 0.48
Cottonseed oil.	Ethyl butyrate. 0.58	Ethyl butyrate. 0.53	Olive oil. Trace.	Glyceryl triacetate. 0.88	Olive oil. 0
Ethyl butyrate.	Methyl benzoate. 0.20	Methyl benzoate. 0.23	Phenyl acetate. 0	Olive oil. 0.35	Cottonseed oil. 0
	Ethyl benzoate. 0	Ethyl benzoate. Trace.	Cottonseed oil. 0	Ethyl butyrate. 0.13	Ethyl acetate. 0
	Phenyl benzoate. 0	Phenyl benzoate. 0	Ethyl benzoate. 0	Methyl benzoate. 0	Methyl benzoate. 0
			Ethyl butyrate. 0		Ethyl butyrate. 0

* Figures indicate extent of action per 0.01 gm. molecule of ester used.

† Falk (1917).

Comparing the orders in which the various esters are hydrolyzed by the different ester-hydrolyzing substances described in the work of Falk with those reported here, marked differences are found. While the work of Falk shows that both ethyl imido-benzoate and glycylglycine exhibit almost the same selective activity in point of order towards different esters, the results with the alkali-treated proteins show that there are marked differences in the case of different proteins. It has been suggested by Falk that the action of the proteins treated with alkali is due to the presence of an enol-lactim structure. It is probably also true that the differences in activity noted with the different proteins is due to the presence of different groupings attached to the sides of the enol-lactim linkage. Such an explanation affords a chemical basis for the various specificities of enzymes for their substrata, the differences in activity being quantitative rather than qualitative in character.

The time of incubation with the ester which was necessary for maximum action was 48 hours.

In the case of casein where hydrolysis was brought about by digestion with papain, the solution possessed no activity, thus lending support to the view that the activity of the solution is not dependent upon products of hydrolysis alone.

The action of the alkaline solutions of casein, gelatin, and egg albumen was tried on a number of different esters. Each solution showed an order of activity peculiar to that solution.

It was not possible to inactivate the solutions by boiling.

The following proteins when acted upon by alkali showed ester-hydrolyzing properties:

Casein.	Egg globulin.
Gelatin.	Pseudoglobulin.
Egg albumen.	Euglobulin.
Castor bean globulin.	Soy bean globulin.
Zein.	Soy bean glutelin.
Phaseolin.	Gliadin.
Edestin.	Lactalbumin.
Ovitellin.	

SUMMARY.

Proteins when treated with alkali yield substances which have the power to accelerate hydrolysis of esters. The effect of the concentration of the alkali in producing these ester-hydrolyzing substances was studied, and for casein, gelatin, and egg albumen 3 N alkali seemed to produce solutions of highest activity.

These solutions showed an optimum hydrogen ion concentration for their activities. They all showed greater activity at a concentration of the hydrogen ion less than 10^{-7} N or they are more active in a slightly alkaline solution.

The time and temperature at which the alkali stood in contact with the protein did not seem to make much difference in the activity of the solution except where the temperature was quite high (80°C.).

When hydrolysis of the protein was accomplished by acid instead of alkali the solution did not possess ester-hydrolyzing properties.

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THE VALUE OF THE YEAST VITAMINE FRACTION AS A SUPPLEMENT TO A RICE DIET.

BY A. D. EMMETT AND L. H. McKIM.

(From the Research Laboratory of Parke, Davis and Company, Detroit.)

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In the present paper, the authors report the first of a series of investigations that has to deal more particularly with the efficiency of the vitamins from autolyzed brewer's yeast, in respect to their value as an *adjuvant* to a diet that has been shown to be deficient in some particular vitamin. Thus, while it is known from the work of Chamberlain, Vedder, Funk, Suzuki, Eykmann, Williams, Seidell and others, that polished rice as the sole article of diet, in the case of pigeons and man, will produce typical polyneuritis and beri-beri, respectively, and that these dietary deficiency diseases can be treated effectively by giving a dose of the vitamin fraction from brewer's yeast, *it is also pertinent to know how effective a rational treatment with this same vitamin will be as a therapeutic agent in convalescent subjects.* Stating this in another way, using the same illustration, will the supplementing of the polished rice diet with the yeast vitamin (where pigeons have been cured of polyneuritis) not only prevent the recurrence of the disease, *but will it bring about as full a recovery, measured by the gain in body weight, as a diet that is known to be complete in respect to the particular vitamins involved?*

Hopkins, Stepp, Funk and coworkers, Osborne and Mendel, McCollum and coworkers, Drummond (1), and others have given abundant evidence of the importance of accessories in the normal dietary. Funk (2) found when polished rice was supplemented with a crystalline vitamin prepared from yeast that it was a complete food in the sense that the diet not only prevented the onset of polyneuritis but brought about a gain in weight. These tests extended over only 29 days. He concluded that there was no justification for the belief that there might be two vitamins,—one for curing polyneuritis, and one for maintaining weight. Funk, Lyle, and McCaskey (3) report a study where they endeavored to determine the nutritive value

of yeast, polished rice, and white bread from the standpoint of the influence of vitamins upon the metabolism of man. It was found that the value of dried yeast as a source of protein was not very good. The high content of the purines in the yeast caused a noticeable rise in uric acid in the blood, and showed definitely that yeast should not be used too freely as a substitute for other foods. Further, when the vitamin- and purine-free diet was supplemented with a vitamin preparation, made by Seidell's method (4), there seemed to have been no apparent effect produced on the nitrogen balance. The authors state that this may have been due to the shortness of the metabolism period or to the fact that the vitamin preparation was not sufficiently active. They found that a vitamin-free diet of white bread and polished rice required more nitrogen than a diet of whole wheat bread or potato to produce a positive nitrogen balance.

In the particular case of polished rice, McCollum and Davis (1) have shown, in feeding young rats, that it is necessary for normal growth, to supplement the diet with both the water-soluble and fat-soluble accessories or vitamins, even though the protein, carbohydrate, and salts in the ration are liberal in amount. McCollum and Kennedy (5) report in their work on polyneuritis in pigeons that the water-soluble accessory is directly concerned in the prevention and cure of this disease and that the fat-soluble fraction is related to maintenance.

Funk and Cooper both demonstrated that autolyzing yeast increased its value as a curative agent for polyneuritis. Seidell (6) has confirmed this more recently. Funk and Macallum (7) believe that autolyzed yeast contains at least two vitamins,—one which will cure polyneuritis and the other which will stimulate growth. They found that the addition of this yeast, along with butter fat, to a ration accelerated growth in rats; that when lard was introduced in place of butter fat no growth resulted if the yeast was dried, but if the yeast was moist, growth resulted, although to a lesser degree than with the butter fat; and it seemed that the growth-promoting substance in yeast was practically identical with the polyneuritic vitamin, except that it required more of the former to bring about growth than of the latter to produce a cure.

Seidell fed pigeons a diet of polished rice and added to it an autolyzed yeast vitamin preparation, made in accordance with his method, using Lloyd's anhydrous aluminum silicate as the adsorptive agent. He found with this supplement that normal pigeons when put upon this diet from the first showed no bad effects but in some cases they made slow but gradual gains in weight over a period of 60 days. Williams and Seidell (8) were able to separate a crystalline product from the Seidell yeast vitamin that contained an active principle. This material was curative, and in administering it to polyneuritic pigeons it brought about gains in weight on a polished rice diet. The gains amounted to about 80 per cent of the initial weight in tests covering 80 days. However, these gains took place during the first 25 to 30 days following the treatment, after which the weight remained practically constant. In one case, a polyneuritic pigeon regained its original weight in 30 days but an unusually large dose (50 mg.) of the

crystalline material was given. In another report, Seidell showed that the autolyzed yeast filtrate will stimulate an appreciable gain in weight in the case of a normal pigeon if given in sufficiently large amounts,—much more than that needed for curing pigeons of polyneuritis. He estimated that the diet of pigeons should contain a little less than 0.0033 per cent of vitamine.

Funk (9) in considering the dietary factors in relation to growth states that while yeast promotes growth, the Seidell vitamine preparation from autolyzed yeast with Lloyd's reagent was greatly deficient in the growth stimulant and has lost some of its antiscorbutic property.

EXPERIMENTAL.

The pigeons, with the exception of a few which were kept for normal controls, were put upon polished rice as the sole food, along with distilled water. At first, the birds on the polished rice were forcibly fed after they began to show a gradual loss of appetite. Later, this procedure was discontinued as it appeared in comparison with the pigeons which were not forcibly fed, that the additional labor involved was possibly not justified. We are not fully convinced, however, at this time, that this is true in all cases, inasmuch as the factor of inanition in some cases seemed to introduce complications which rendered some pigeons more susceptible to infection.

When the pigeons came down with typical polyneuritis and reached the critical stage, they were treated with the Seidell autolyzed yeast vitamine. Special care was used to keep the birds, during this period of the disease and treatment, isolated in separate cages, as it was evident to us that absolute quiet was a factor which tended to hasten the recovery of the pigeons. For 24 hours therefore after treatment, the pigeons were handled only when absolutely necessary, given water *ad libitum*, and only a very limited amount of feed.

The diet, after the treatment, consisted of one of the following: polished rice with vitamine, shelled corn, brown or natural rice, brown rice with vitamine, barley, unhulled oats, and hulled oats. In general, one of these diets was fed until the pigeons seemed to cease to gain in weight, when another diet was substituted for it. No attempt was made to adjust the mineral deficiencies in the different foods.

Preparation of the Yeast Vitamine.—In general, the method of Seidell was followed in separating the vitamine from the autolyzed

brewer's yeast filtrate. We introduced certain modifications relating to the removal of the liquor from the yeast, the drying of the yeast, the length of time of autolyzing, etc.

Several forms of aluminum silicate were tested as to their absorptive power for vitamine, among them being infusorial earth; the trade products "filtercel," and "celite B;" the usual form of fullers' earth; and the special form of fullers' earth, called Lloyd's reagent. Lloyd's reagent was taken as the standard. The activated vitamine materials were made into tablets, a definite amount of lactose being added so as to provide the necessary excipient to give them the proper body.

DISCUSSION.

Effect of the Reagents in Producing Polyneuritis.—In Chart 1 four typical curves are shown representing the effect of feeding pigeons polished rice supplemented with one of the following reagents: Lloyd's special fullers' earth, ordinary fullers' earth, and lactose. The results show conclusively that these reagents had no influence in retarding or hastening the onset of the polyneuritic attack or in effecting the loss in weight. The difference in the length of time that was required to bring about the typical stage of polyneuritis was no greater than the variations in time that we have found with a large number of pigeons that we have fed. The time of the onset depends in part, it seems, upon the thriftiness of the pigeons at the start, the age, the temperature of the room, and whether the birds are forcibly fed or not.

Comparative Value of the Lloyd's Reagent and Fullers' Earth Vitamine Preparations.—In Chart 2, Pigeons 18 and 23 represent two groups of birds that were fed polished rice alone. No. 23 was treated for polyneuritis with the Lloyd's reagent yeast vitamine (L.R.) and No. 18 with the fullers' earth preparation (F.E.). The curves illustrate very clearly the comparative value of the two vitamine preparations, showing that they were apparently of equal strength, when given in the same amounts. A number of similar tests indicated definitely that one preparation was as good as the other and that both were as effective in the treatment of pigeons as the autolyzed yeast filtrate itself.

After treatment, these respective birds were placed on the polished rice diet, supplemented with the *same* kind of vitamine

preparation that was used in bringing about the recovery from the attack of polyneuritis. Later, the pigeons were put on barley without vitamine, then on oats, and finally on hulled oats. Throughout this whole cycle, the two respective curves followed in almost the same direction.

Influence of the Different Dietary Treatments on Polyneuritic Pigeons.—The effect of feeding the pigeons, after they were brought down to the typical and fulminating stage of polyneuritis and then treated with the vitamine preparation, is shown in Charts 3 and 4.

Other instances of the effect upon the weight curves of feeding pigeons polished rice along with the Seidell autolyzed yeast vitamine (F.E.), are shown in the case of Nos. 37 and 52, Chart 1; and also, of Nos. 10 and 15, after their recovery from the second attack of polyneuritis, Chart 4.

It was assumed that the quantity of yeast vitamine added in supplementing the rice diets ought not to be more than the amount used in the treatment for the typical attack of polyneuritis, since this amount was always as great as the quantity contained in an equal weight of brown or natural rice. It seemed to us that if a much larger amount of this particular vitamine was needed to bring about as good a recovery as could be obtained by feeding a diet of brown rice or other vitamine-containing grains, then we were dealing here with a vitamine preparation that was *qualitatively* different from that which was removed in the polishing of the rice, the difference being due either to the vitamine contained in the autolyzed yeast filtrate or to the alteration of this vitamine by the method which was used in separating it from the yeast filtrate. The amount used was equivalent to 0.03 to 0.06 gm. of the Seidell vitamine per day.

To summarize, after making allowance for the influence of confinement and environment upon the weight of the pigeons, the effect of the dietary treatments, *after recovery from a typical attack of polyneuritis*, was as follows:

(a) Polished rice plus the autolyzed yeast vitamine of Seidell prevented a recurrence of polyneuritis, and brought about a partial gain in weight, which after 75 to 90 days from date of recovery began to decrease and then came to a more or less constant level. The birds were active and thrifty throughout.

(b) Brown rice, as the sole diet, brought about very good gains. In some cases the pigeons not only recovered all that they lost in coming down with polyneuritis, but went beyond their initial weight at the time of being put in the cage. It is barely possible, although doubtful in the light of our present knowledge, that some of the differences between the effect of this diet and the preceding one (a) may have been due in part to the removal of phosphorus in the polishing of the rice. This point is being studied.

(c) Brown rice plus the yeast vitamine produced the best gains of any diet tried. The yeast vitamine acted as a stimulant here, even though there was an ample amount of vitamine in the brown rice alone to produce normal gains.

(d) Shelled corn was an efficient diet, producing gains that were greater than those obtained with the polished rice plus the vitamine, but less than those with the brown rice alone.

(e) Barley brought about fair gains, better than the polished rice plus vitamine, but its effect was soon lost, the pigeons declining rapidly in weight.

(f) Unhulled oats proved to be unsatisfactory as a normal diet for pigeons.

(g) Hulled oats were much better than the unhulled.

CONCLUSIONS.

From these studies, which have extended over a much longer period of time than any of those previously reported on this subject, it appears:

1. That ordinary fullers' earth, ground to a definite fineness, adsorbs the yeast vitamine fraction from the autolyzed yeast filtrate as readily as the special form of fullers' earth, called Lloyd's reagent. Further, the *Kieselguhrs* (infusorial earth, "celite B," and "filtercel") do not adsorb the yeast vitamine.

2. That these two forms of activated fullers' earth are equally potent and very efficient as therapeutic agents in the treatment for typical attacks of avian polyneuritis.

3. That silicates in the form of ordinary fullers' earth, Lloyd's reagent, or infusorial earth, when added to a polished rice diet, do not inhibit or accelerate the onset of avian polyneuritis;

and that lactose, used in making the vitamine tablets, is also inert in this respect.

4. That (a) the activated fullers' earth when given as a *rational* supplement to a polished or a brown rice diet, acts as a partial stimulant to increase the weight of the treated polyneuritic pigeons; (b) it does not, however, in the case of the polished rice, accelerate the increase in weight to anything like that which is produced under similar conditions with brown rice alone, corn, barley, or hulled oats; and (c) comparatively speaking, this activated yeast vitamine is not a complete supplement to a polished rice diet.

5. That there are apparently two so called vitaminases associated with rice polishing, one which cures polyneuritis and one which produces weight, and of these two, the Seidell yeast vitamine preparation contains chiefly the curative fraction, along with a small per cent of the other.

6. Finally, it is evident, that while this activated yeast vitamine product is a valuable adjuvant to the diet in the case of convalescents from avian polyneuritis, yet, for the best results, the diet should, in addition, be made up in part at least of vitamine-containing foods, not for the purpose of preventing the recurrence of typical attacks of this dietary deficiency disease (for the preparation is able to do this) but with the object of bringing about normal gains in weight and *complete* recovery.

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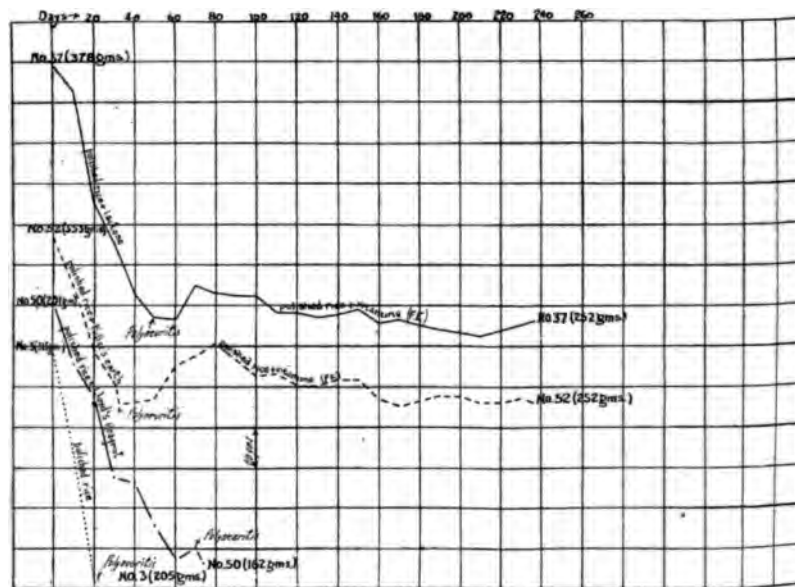


CHART 1. The curves show that the supplementing of the polished rice diet with lactose, or fullers' earth, or the special form of fullers' earth called Lloyd's reagent, has no effect upon accelerating or retarding the onset of polyneuritis. After the 55th day, the curves for Pigeons 37 and 52 illustrate the influence of feeding convalescent polyneuritic pigeons upon polished rice and yeast vitamine (Seidell).

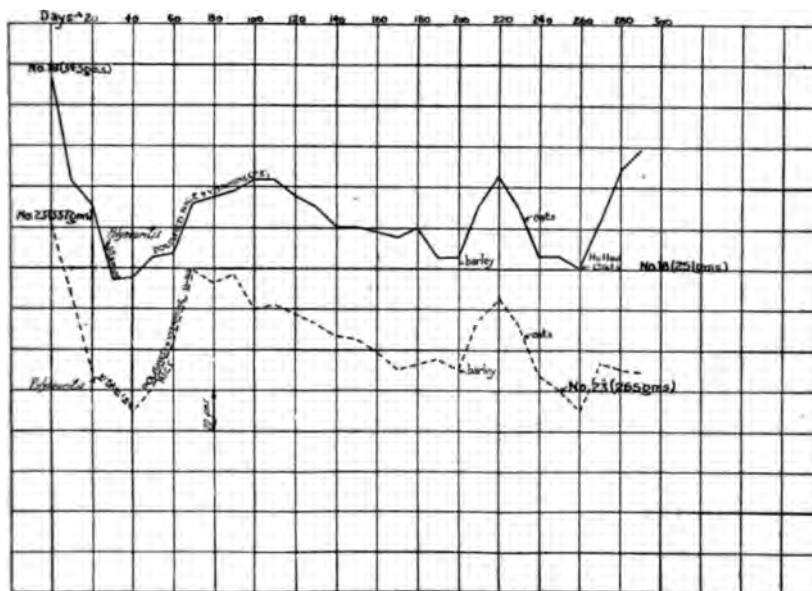


CHART 2. The two weight curves indicate how closely the vitamine preparations, made with the usual fullers' earth and the special form, Lloyd's reagent, compare as to their curative and therapeutic value. They also represent the comparative value of the rations of polished rice with vitamine, barley alone, oats alone, and hulled oats alone.

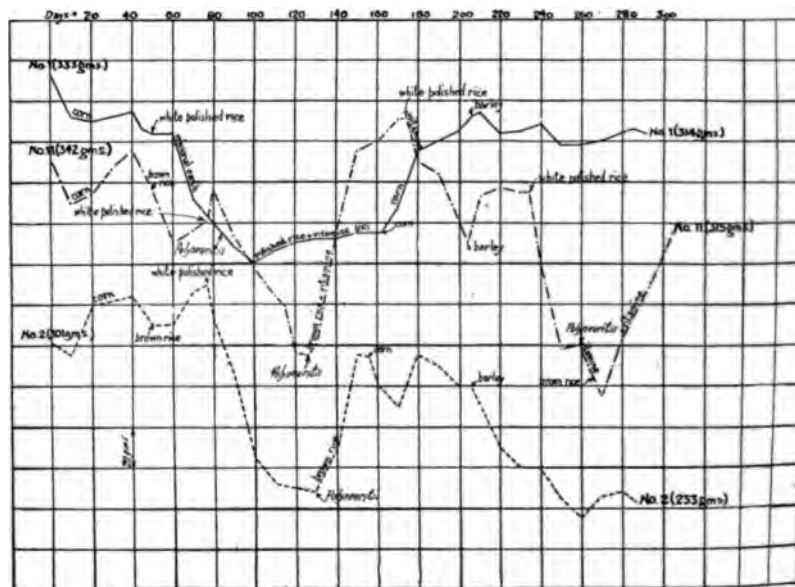
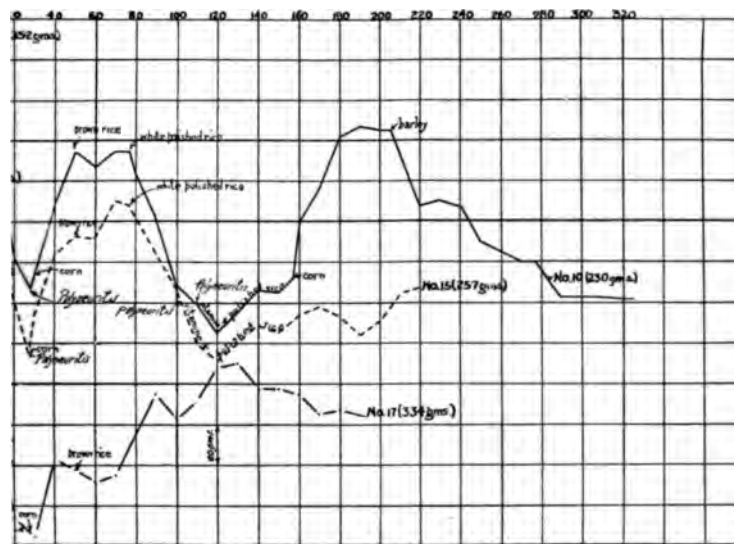


CHART 3. These weight curves show first, the effect of confinement in the cage and environment over the first 55 to 80 days; second, the neutral effect of infusorial earth (Pigeon 1); third, the therapeutic value of yeast vitamine as a supplement to the diets,—polished rice alone, polished rice with yeast vitamine, brown or natural rice with yeast vitamine, compared with brown rice alone, corn alone, and barley alone.



ART 4. The curves show first, the typical decline in weight with a
 red rice diet; second, the comparative nutritive value of brown or
 al rice and corn when fed to pigeons after vitamine treatment for
 al polyneuritis; and third, the therapeutic value of supplementing
 amine-free diet (polished rice) with yeast vitamine compared with
 ine-containing foods, brown rice, corn, and barley.

ANIMAL CALORIMETRY.

THE INTERRELATION BETWEEN DIET AND BODY CONDITION AND THE ENERGY PRODUCTION DURING MECHANICAL WORK.*

THIRTEENTH PAPER.

BY R. J. ANDERSON† AND GRAHAM LUSK.

(From the Physiological Laboratory, Cornell University Medical College,
New York City.)

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INTRODUCTION.

In the first and second editions of *The Elements of the Science of Nutrition*¹ a calculation was published which was based upon experimental work of Voit, accomplished in 1866, and which suggested that when a given quantity of mechanical work was performed the height of the heat production was the same whether a man were fasting or had partaken liberally of a mixed diet.

* A brief abstract of this work was published in the *Proc. Nat. Acad. Sc.*, 1917, iii, 386. The conclusions there presented have been slightly modified.

† Of the Chemical Laboratory of the New York Experiment Station, Geneva.

¹ Lusk, G., *The Elements of the Science of Nutrition*, Philadelphia, and London, 1906, 2nd edition, 1909; *Ernährung und Stoffwechsel*, Wiesbaden, translation of 2nd edition, 1910.

The problem was investigated by Rubner² in experiments upon a man weighing 61 to 63 kilos. The metabolism of the man at rest and without food was measured as being 1,976 calories and was only slightly greater, 2,023 calories, during a day characterized by the ingestion of 600 gm. of sucrose dissolved in 3,000 gm. of water. When, however, mechanical work, amounting to the equivalent of 100,000 kilogrammeters, was accomplished during a day when this same quantity of sugar was taken, the metabolism rose to 2,868, an increase of 845 calories. When a large amount of meat was given to the man on a resting day the heat production was 2,515 calories, an increase of 539 calories, caused by the specific dynamic action of the meat metabolized. When mechanical work equivalent to 100,000 kilogrammeters was executed on a day when meat was taken as above described, the metabolism rose to 3,370 calories, an increase due to work of 855 calories above the level reached when meat was given on a resting day. Rubner noted that mechanical work could be performed with much greater ease after cane sugar was given than after meat was given, and also that the amount of sweat produced was largely increased in the latter instance.

These experiments indicated that there was a summation of the extra energy production due to the specific dynamic action of meat and the energy produced as the accompaniment of mechanical work.

The criticism may be made that 1,976 calories reported for the resting man without food is too high and was associated with considerable muscular movement. If the basal metabolism of the man were assumed to be 70 calories per hour—a high figure for his weight—he would have produced only 1,680 daily instead of 1,976, as reported. This consideration would throw doubt upon the validity of the quantity of the increase in heat production after giving glucose, for example.

Benedict and Murschhauser³ also state that in man there is a summation of the two energy increments due respectively to work and food ingestion.

² Rubner, M., *Sitzungsber. preus. Akad. Wissensch.*, 1910, xvi, 316.

³ Benedict, F. G., and Murschhauser, H., *Energy Transformations during Horizontal Walking*, *Carnegie Institution of Washington, Publication 231*, 1915, 91.

It seemed desirable to extend these experiments to the dog, an animal in which dietary control is more readily obtained than in the human being.

Problems.

The experiments deal with the influence of meat ingestion or glucose ingestion upon the heat production when a dog runs at the rate of about 3 miles an hour, and also the influence of fasting upon the quantity of energy required to do a given amount of mechanical work. Incidentally, the respiratory quotient obtained from the almost exclusive oxidation of body fat during exercise after long fasting was noted, and the influence of food given at the end of a fasting period was observed.

Methods.

A treadmill was constructed by Mr. J. A. Riche to fit within the calorimeter built by Williams⁴ for this laboratory. The treadmill was operated by a motor outside the calorimeter. Each revolution of the wheel operating the treadmill represented a forward movement of the platform of 0.458 meter and every 1,000 revolutions produced 0.5 calorie by friction in the box of the calorimeter, as was experimentally determined. The rate of revolution of the treadmill was very even from hour to hour. It was, however, impossible to determine the heat production by the treadmill when the dog was running upon it and, by the intermittent application of his weight, adding to the friction of its parts. It was therefore impossible to compare the results of direct and indirect calorimetry. At no time did the dog show signs of fatigue or of thirst after the completion of the run on the treadmill.

A factor which caused great annoyance was that the dog (No. XV, a white bull-terrier, female), while running, would never hold her urine throughout an experiment. The figures given for the nitrogen elimination on the days when work was done are those obtained during periods of similar dietary conditions when the dog was quiet. Since it has been frequently shown that mechanical work is without influence upon protein metabolism, this factor

⁴ Williams, H. B., *J. Biol. Chem.*, 1912, xii, 317.

would involve little error, except that possibly mechanical work might have affected the rate of the absorption of protein through a more rapid circulation of blood in the gastrointestinal canal.⁵ A separate attempt to determine this relationship during the 4th and 5th hours after meat ingestion failed because the dog urinated every 3 to 5 minutes. This factor made the determinations of the water elimination valueless; hence it is impossible to give the percentile quantity of heat lost from the running dog through the evaporation of water. In spite of these limitations the results of the respiratory metabolism are clearly of significance.

The composition of the "standard diet" has been given elsewhere.⁶ As a rule the dog ran in the calorimeter for $\frac{3}{4}$ of an hour before the commencement of an observation. In certain instances a preliminary observation of the basal metabolism was made and then about 2 minutes after the close of the preliminary period the treadmill was started. This accounts for the lesser number of revolutions of the treadmill in certain first hours of the experiments with work. These hours were not included in the calculations.

The Basal Metabolism.

In a preliminary experimental *first series* the *basal metabolism* of the resting dog was found to be 17.5 calories per hour (Experiment 4, Table X). The basal metabolism is determined about

TABLE I.
Basal Metabolism.

Date.	Experiment No.	Indirect calorimetry.	R. Q.
1917		cal.	
Mar. 2.....	10	19.0	0.94
" 8.....	14	17.7	0.95
		16.7	0.83
		18.6	0.77
" 17.....	21	15.8	0.86
" 22.....	23	15.5	0.80
		17.2	0.86

⁵ A paper bearing upon this subject is in course of preparation.

⁶ Lusk, *J. Biol. Chem.*, 1912-13, xiii, 185.

18 hours after administration of the standard diet when a dog is quietly resting in the calorimeter at a temperature of 26°.

In the *second series* of experiments the basal metabolism was 17.2 calories, as calculated from the average of the hours of experimentation in Table X, second series.

Mechanical Work 18 Hours after Food Ingestion.

The quota of heat production due to muscular activity was found by subtracting the basal metabolism of the dog from the metabolism determined while the dog was at work, the experiment being done about 18 hours after administration of the standard diet. Table II is arranged to show the data obtained, the results being given in the order of increasing speed.

TABLE II.
The Influence of Mechanical Work 18 Hours after Food Ingestion.

Date.	Experiment No.	Weight of dog.	Indirect calorimetry.	R. Q.	Work in distance traveled.		Calories above the basal per 1,000 meters traveled.	Work in kg.-m. to move 1 kg. 1 meter.
1917		kg.	cal.		miles	meters		
Jan. 5.....	2	8.0	60.8	0.78	2.48	3,925	11.0	0.585
Feb. 28.....	8	9.0	70.0	0.81	2.67	4,300	12.3	0.580
Mar. 13.....	18	8.9	74.8	0.75	2.90	4,688	12.2	0.585
“ 23.....	24	9.2	76.7	0.74	2.92	4,717	12.6	0.582
“ 27.....	27	9.4	76.7	0.81	2.92	4,718	12.6	0.570
	13	9.0	76.1	0.79	2.98	4,806	12.2	0.578
				0.78				0.580

Maximum variation = ± 1.7 per cent.

The analysis of Table II shows that the forward motion of this dog was accomplished at the expenditure of energy of metabolism equal to 0.580 kilogrammeter per kilo of body substance moved 1 meter, and that this factor showed a variation of less than 2 per cent although the weight of the dog varied 17 per cent and the rate traveled varied 20 per cent.

The energy has been given in terms of kilogrammeters because this form was used in many communications from Zuntz's laboratory.

Thus, Frentzel and Reach⁷ report that it requires 0.501 kilogram-meter of energy to move 1 kilo of a dog weighing 26.9 kilos 1 meter horizontally when the dog was traveling at the rate of 4,714 meters per hour. Slowtzoff⁸ states that a dog weighing 5.5 kilos required 1.14 kilogram-meters of energy to accomplish the same unit of work. These two experiments show no harmonious relationship with the value of 0.580 kilogram-meter of energy obtained from Dog XV weighing 9 kilos. It is probable that the anatomical structure and muscular development of the dog bear some relation to its efficiency as a machine.

When one considers the total metabolism in relation to the weight of the dog, it appears that it requires a lesser amount of energy to move the dog's body horizontally when it is thin than when it is better nourished. This appears from arranging the data as in Table III.

TABLE III.

Experiment No.	Weight.	Calories above the basal per 1,000 meters traveled.
	<i>kg.</i>	
2	8.0	11.0
18	8.9	12.2
13	9.0	12.2
8	9.0	12.3
24	9.2	12.6
27	9.4	12.6

A gain of body weight of 15 per cent brought about a corresponding gain of 15 per cent in the energy requirement for moving the body horizontally.

Mechanical Work after Glucose Ingestion.

It has been shown that when 70 gm. of glucose are given to a dog the heat production rises about 30 per cent above the level of the basal metabolism and this continues during 4 hours after the ingestion of the material. In Dog XV the rise in metabolism after giving 70 gm. of glucose did not appear to be as great as usual, as is shown in Table IV.

⁷ Frentzel, J., and Reach, F., *Arch. ges. Physiol.*, 1900-01, lxxxiii, 404.

⁸ Slowtzoff, B., *Arch. ges. Physiol.*, 1903, xcv, 190.

TABLE IV.
Increases in Metabolism after Giving Glucose 70 Gm.

Date.	Experiment No.	Weight.	Indirect calorimetry.	R. Q.	Percentage increase above the basal.
1917		kg.	cal.		
Mar. 1.....	9	9.3	19.88	1.04	+16
" 16.....	20	9.3	20.95	1.07	+22

The total metabolism rose 2.7 and 3.7 calories above the basal as the result of giving 70 gm. of glucose in water warmed to the body temperature.

When the dog was given glucose, 70 or 100 gm., and then caused to run on the treadmill during those hours in which the basal metabolism would have been increased by the glucose ingestion, the heat production was almost the same as when the animal ran the same distance 18 hours after food ingestion.

These relations are set forth in Table V.

TABLE V.
Comparison of the Effect of the Same Amount of Mechanical Work 18 Hours after Food and during the Hours Immediately Following the Ingestion of Glucose.

Date.	Experiment No.	Weight of dog.	Food.	Indirect calorimetry.	R. Q.	Work in distance traveled.		Calories above the basal per 1,000 meters traveled.	Work in kg.-m. to move 1 kg. 1 meter.
1917		kg.		cal.		miles	meters		
Jan. 5	2	8.0	No food.	60.8	0.78	2.45	3,925	11.0	0.585
	3	8.35	Glucose, 70 gm.	62.3	0.98	2.44	3,936	11.1	0.579
Mar. 15	19	9.6	" 70 "	77.1	0.92	2.96	4,771	12.5	0.555
" 23	24	9.3	No food.	76.7	0.74	2.92	4,717	12.6	0.582
" 26	26	9.7	Glucose, 100 gm.	76.8	0.95	2.94	4,737	12.5	0.550

Experiment 24 is reported for comparison with Experiment 19 instead of Experiment 18, of nearer date, because the weight of the dog in the last named basal experiment was reduced on account of the exclusive meat diet of the day preceding.

When 70 gm. of glucose were given to the dog the weight of the water solution administered was 280 gm. and when 100

gm. of glucose were given the added weight was 350 gm. These values were the cause of the apparent increase in the weight of the dog on the days when glucose was ingested.

It is apparent from the table given above that *when mechanical work is accomplished during the hours following a large ingestion of glucose, the metabolism rises to about the same height as when the same amount of work is done during a period when the gastrointestinal tract is free from food.* The amount of energy necessary for the dog to transport itself 1,000 meters horizontally is the same under both sets of circumstances.

Curiously enough, in the second series of experiments there appears to be no added increment of energy needed to transport the weight of the solution ingested. If it be considered that this factor must require energy for its accomplishment, then *one may conclude from the evidence that the forward movement of the dog's own weight may be executed with even less expenditure of energy in the presence of abundant carbohydrate food than when no food is taken.* Since, in the two instances, the energy requirements for the forward motion of 1 kilo of body weight 1 meter are, respectively, 0.550 and 0.580, it appears that after glucose ingestion mechanical work may be performed at an expense of 5 per cent less energy than when no food is taken.

The respiratory quotients do not rise above unity in any of these experiments. To furnish 75 calories in the form of glucose in Experiments 19 and 26 would have required an absorption of glucose from the intestine at the rate of 20 gm. per hour. Very probably this was above the limit of rapidity of absorption. Under these circumstances there could have been no condition of *carbohydrate plethora*, but the particles of glucose must have been used for the accomplishment of work as they entered the circulation.

Mechanical Work after Meat Ingestion.

The influence of the ingestion of meat upon the metabolism of a dog executing mechanical work is in exact accord with Rubner's experiments upon a man under like conditions.

Meat was given to the dog in the form of finely chopped beef heart and, during the 4th and 5th hours thereafter, the metabolism of the animal was determined while he was running. The

experiment, therefore, was carried on at the time of the maximum effect of the specific dynamic action of the meat.

The results are summarized in Table VI.

TABLE VI.
The Metabolism during Mechanical Work Executed 4 and 5 Hours after the Ingestion of Meat.

Date.	Experiment No.	Weight. <i>kg.</i>	Food.	Indirect calorimetry. <i>cal.</i>	R. Q.	Work in distance traveled.		Calories above the basal per 1,000 meters traveled.	Work in <i>kg.-m.</i> to move 1 <i>kg.</i> 1 meter.
						<i>miles</i>	<i>meters</i>		
1917									
Jan. 5	2	8.0	No food.	60.8	0.78	2.45	3,925	11.0	0.585
" 11	6	9.25	Meat, 700 gm.	82.1	0.82	2.55	4,101	15.8	0.724
Mar. 6	13	9.0	No food.	76.1	0.79	2.98	4,806	12.2	0.578
" 12	17	9.6	Meat, 750 gm.	92.4	0.80	2.91	4,704	16.0	0.708
" 10	16	9.5	" 750 "	30.0*	0.80				

* Dog resting.

In both Experiments 2 and 17 the quantity of energy necessary to move the dog's weight horizontally was very greatly increased. In the first instance it required 24 and in the second 22 per cent more energy to move 1 kilo 1 meter through space than it required when no meat was taken.

In the experiments of the second series one may deduct the value of the basal metabolism (17.2 calories) from that of the work period when no food was taken (76.1 calories) and arrive at the value 58.9 calories as the measure of the power required to do the work. If, now, one deducts the value in calories of the 5th and 6th hours after meat ingestion (30.0 calories) from that of the corresponding period of work after taking meat, one obtains the value 62.4 calories as the measure of the energy actually expended for the work accomplished. If it required 62.4 calories to move the dog weighing 9.6 kilos 4,704 meters, it may be calculated that the equivalent of 0.587 kilogrammeter of energy was necessary for the horizontal movement of 1 kilo of body weight 1 meter. This falls within the error limit for a similar factor where work is done and the gastrointestinal tract is free from food.

It is apparent, therefore, that *when mechanical work is accomplished after high protein ingestion, there is an exact summation of the increment due to the specific dynamic action of meat and that due to energy necessary for the mechanical work involved.*

Mechanical Work after the Ingestion of Alanine.—It was deemed advisable to confirm the results obtained after meat ingestion by making a series of similar experiments after giving 20 gm. of alanine, the specific dynamic action of which is known.⁹ The method for calculating the results in the alanine experiments will be found elsewhere.¹⁰ Table VII gives the results.

TABLE VII.
Metabolism during Mechanical Work Executed 3 and 4 Hours after the Ingestion of Alanine, 20 Gm.

Date.	Experiment No.	Weight.	Food.	Indirect calorimetry.	R. Q.	Work in distance traveled.		Calories above the basal per 1,000 meters traveled.	Work in kg.-m. to move 1 kg. 1 meter.
1917		kg.		cal.		miles	meters		
Mar. 23	24	9.3	No food.	76.7	0.74	2.92	4,717	12.6	0.582
" 20	22	9.3	Alanine, 20 gm.	82.0	0.78	2.96	4,777	13.6	0.620
" 24	25	9.4	" 20 "	21.0*	0.84				

* Dog resting.

If one subtracts the metabolism after giving alanine from the metabolism found while mechanical work was being performed after alanine administration, one obtains the value of 61 calories as the energy necessary to move the body weight, or 9.3 kilos 4,777 meters. This is the equivalent of **0.583** kilogrammeter of energy for the movement of 1 kilo of body weight 1 meter.

It is evident from this experiment that *alanine acts as a stimulant to metabolism during a period of mechanical work in such a way that there is a summation between the increment of extra heat due to its metabolism and the extra heat evolved for the production of work.* The extra heat due to the specific dynamic action of alanine can-

⁹ Lusk, *J. Biol. Chem.*, 1912-13, xiii, 155.

¹⁰ Lusk, *J. Biol. Chem.*, 1915, xx, 571.

not be due to a plethora of extra metabolites because in these experiments comparatively little of the heat production could have been derived from alanine (Table X) but it must have been due to a specific stimulus. The primary metabolism product of alanine is believed to be either lactic acid or pyruvic acid. The contrast between the action of alanine, 20 gm. (which is convertible into 20 gm. of glucose) and of glucose 100 gm., is clear and sharp cut, the intermediary acid products of the first constituting a direct stimulus to metabolism while the metabolites of glucose do not.

Mechanical Work during Fasting.

Before a run in a hunt for deer, dogs were fasted for 5 days, when such sport was allowed, and this without prejudice to the powers of the animal. Dog XV, on the 13th day of fasting, after a run of about 9 miles during 3 successive hours, jumped out of the calorimeter, ran around the room, and in friendly fashion, pawed one of the observing staff. It was at no time exhausted by the work upon the treadmill.

The fast of Dog XV may be described as an "interrupted fast." That is to say, after 8 days of fasting the dog was given, during two "intermediate days" at 5 p.m., a half portion of the "standard diet." This half portion amounted to: meat, 50 gm.; biscuit meal, 50 gm.; lard, 10 gm.; bone ash, 10 gm.; calories 344 daily or 14.3 per hour. The calories administered were exactly the number produced per hour by the quiet, resting, fasting animal at the time and, therefore, could not have contributed caloric value to its body substance. The second division of this interrupted fast began as the 9th day of fasting and was continued until the 15th day. The fast was finally interrupted by giving half portions of the standard diet and then the full standard diet, in order to observe whether, with the full standard diet, the metabolism of the animal would quickly return to its former level.

Table VIII gives the results obtained.

This experiment shows how a dog weighing about 9.2 kilos and manifesting a basal metabolism of 17.2 calories per hour may be reduced in weight to 7.4 kilos on the 13th day of fasting, a loss of 20 per cent; and in heat production to 12.4 cal-

TABLE VIII.

The Influence of Mechanical Work upon the Metabolism in Fasting; and the Influence of Food Ingestion after Fasting.

Date.	Experiment No.	Weight. <i>kg.</i>	Day of fast.	Indirect calorimetry. <i>cal.</i>	R. Q.	Work in distance traveled.		Calories above the basal per 1,000 meters traveled.	Work in kg.-m. to move 1 kg. 1 meter.
						<i>miles</i>	<i>meters</i>		
1917									
Mar. 29.....	28	8.75	3	73.7	0.710	2.97	4,796	12.0	0.584
" 30.....	29	8.6	4	16.0	0.74				
" 31.....	30	8.55	5	70.2	0.724*	2.93	4,719	11.5	0.570
Apr. 2.....	31	8.35	7	15.0	0.74				
" 3.....	32	8.1	8	14.3	0.715				
			8	70.8	0.719*	3.11	5,023	11.2	0.587
			†						
" 5.....	33	8.2	9	14.4	0.73				
" 9.....	34	7.6	13	12.4	0.75				
				62.7	0.717*	2.92	4,710	10.6	0.595
" 11.....	35	7.45	15	13.0	0.73				
" 13.....	36	7.6	†	12.6	0.80				
" 14.....	37	7.9	‡	13.0	0.93				
" 21.....	40	8.0	§	13.9	0.88				
Average.....									0.584

* Last hour of mechanical work.

† Half portions of standard diet for 2 days.

‡ Full portion of standard diet on previous day.

§ Standard diet during 8 previous days.

|| Maximum variation = ± 2.4 per cent.

ories, a reduction of 28 per cent. When the dog was in good condition it required 12.5 calories to move the body weight 1,000 meters horizontally, but the emaciated dog required only 10.6 calories to accomplish the same result, a reduction of 15 per cent.

However, when one notes the quantity of energy of metabolism required by the dog to move 1 kilo of body weight 1 meter horizontally during the various days of fasting, one finds an average value of 0.584 kilogrammeter with a maximum variation

of 2.4 per cent. This compares with an average value of 0.580 kilogrammeter of energy required for a similar amount of work by the well nourished dog, as given on page 425. It may therefore be stated that *the quantity of energy required to accomplish a given amount of work is the same whether a dog is in the best nutritive condition or has lost 20 per cent in weight after 13 days of fasting.*

By a reduction of the body weight one may economize in the quantity of the basal metabolism, one may economize in the quantity of food fuel required to move the lesser body weight, but if a given amount of work is to be done it can be accomplished only at the expense of a definite quantity of energy, irrespective of the body weight.

Respiratory Quotients in Work and Fasting.—During the periods of work and starvation the respiratory quotients are especially interesting because they represent, in the last hour of the experiments at least, the combustion of almost pure fat with scarcely more than 3 per cent of the total calories derived from protein.

The calculated non-protein respiratory quotients for successive hours are shown below.

Experiment No.....		30	32	34
Day of fast.....		5	8	13
Respiratory quotient.....	1st hr.		0.740	0.721
“ “	2nd “	0.734	0.727	0.717
“ “	3rd “	0.722	0.717	0.713

In all these experiments there is a fall in the respiratory quotients from hour to hour, the fall being least noticeable in the last experiment on the 13th day of fasting. The higher quotients are due to the utilization of residual stores of glycogen. Lehmann, Mueller, Munk, Senator, and Zuntz¹¹ calculated 0.710 to be the respiratory quotient for lard. Later Zuntz¹² slightly changed the oxygen value so that the quotient became 0.707. Still more

¹¹ Lehmann, C., Mueller, F., Munk, I., Senator, H., and Zuntz, N., *Virchows Arch.*, 1893, cxxxi, suppl., 131.

¹² Zuntz, N., *Arch. ges. Physiol.*, 1897, lxxviii, 201.

recently Zuntz¹³ computed the respiratory quotient for human fat to be 0.713. This is the lowest quotient obtained in the above series and is probably not far removed from the actual respiratory quotient of dog's body fat.

Food Ingestion after Fasting.—The influence of food ingestion after fasting upon the level of the basal metabolism was found to be slight. Thus, when on the 8th day of fasting a half portion of the standard diet, or the energy equivalent of 14.3 calories per hour, was given and this was repeated the next day, the basal metabolism remained unchanged. The two observations were 14.3 and 14.4 calories per hour. The same result was noted after giving half a portion of the standard diet on the 15th day of fasting, and on the following day. Even the administration of the full standard diet failed to raise the metabolism, 18 hours after its ingestion, to a level higher than that which had existed in fasting. *The condition of the body, and not a large influx of food on the day previous, determines the height of the basal metabolism.*

On the conclusion of these experiments the animal was given daily 750 gm. of meat at 10 a.m. and the standard diet at 5 p.m. The animal gained 1.1 kilos in a week and the basal metabolism rose to 17.8 calories per hour. At this time the administration of 750 gm. of meat caused the heat production, during the 5th hour after food ingestion, to rise to 32 calories (Experiment 43) in contrast with a metabolism of 12.4 calories per hour on the 13th day of fasting recorded a month earlier. The increase is one of 158 per cent, which is striking but not unexpected.

Table IX presents the details of urinary analyses, and Table X the data of the respiration experiments.

SUMMARY.

1. A dog, 18 hours after taking a mixed diet and weighing about 9 kilos, moved horizontally on a treadmill at rates varying between 2.5 and 3 miles (3,900 and 4,800 meters respectively) per hour and accomplished this work at the expense of an extra energy production of **0.580** kilogrammeter per kilo of body

¹³ Zuntz, *Lehrbuch der Physiologie des Menschen*, Leipzig, 2d edition, 1913, 644.

weight transported 1 meter. The variation in this factor was less than 2 per cent.

2. The same dog, after taking 70 or 100 gm. of glucose in aqueous solution, accomplished the same amount of work at the expense of extra energy production, corresponding to 0.550 kilogrammeter per kilo of body weight transported 1 meter. It is evident that after carbohydrate ingestion a given amount of work may be accomplished with an amount of energy which is the same or even less than when no food is given. Certainly the inflow of metabolites of glucose induced no extra heat production.

3. When given meat, the dog accomplished the work of running 3 miles an hour at the expenditure of an amount of extra energy above the level of the basal metabolism, amounting to the sum of the extra heat which would have been produced by the specific dynamic action of meat plus an energy increment of 0.587 kilogrammeter for moving 1 kilo of body weight 1 meter.

4. The dog, when given 20 gm. of alanine, accomplished the work of running 3 miles in such a way that the increase above the basal metabolism amounted to a summation of the increment of extra heat due to the specific dynamic action of alanine plus an energy increment of 0.583 kilogrammeter for the horizontal movement of 1 kilo of body weight 1 meter.

The metabolites of meat and alanine, therefore, act differently from those of glucose.

5. After fasting the dog 13 days, until it had lost 20 per cent of its weight, it was found that on various days throughout the fast it required the energy equivalent of 0.584 kilogrammeter to move 1 kilo of body weight 1 meter.

6. It is evident from this that the quantity of energy required to move 1 kilo of body substance 1 meter through space (0.580 kilogrammeter) was a constant factor, which was independent of the condition of the body and was separable from that specific dynamic quota due to the stimulation of the metabolites of protein. Only in the presence of an abundant supply of the metabolites of glucose could it be slightly reduced.

7. The lowest non-protein respiratory quotients found after long running on the treadmill during fasting were 0.717 and 0.713, which probably represent the quotients for body fat.

8. The ingestion in various quantities of a mixed diet during

a fast had no effect upon the basal metabolism of the day following food administration, as compared with the level of metabolism previous to giving the food. The condition of the body and not a large influx of food on the day previous determined the height of metabolism.

We wish to thank Mr. James Evenden for the technical assistance he gave in operating the calorimeter.

TABLE IX.
Urinary Nitrogen—Dog XV.
Second Series.

Date.	Kind of experiment.	Gm. nitrogen eliminated per hour.					
		1st hr.	2nd hr.	3rd hr.	4th hr.	5th hr.	6th hr.
1917							
Feb. 20	1st day of 700 gm. meat.	0.214	0.480	0.847	0.934	0.973	1.026
21	2nd " " 700 " "	0.65*	0.65*	1.19*	1.19*		
27	Basal. Standard diet						
	Feb. 26.	0.198	0.203				
Mar. 1	Glucose metabolism. 70 gm. glucose.	0.103					
2	Basal. Standard diet	0.108					
	Mar. 1.						
8	Basal. Standard diet	0.098					
	Mar. 7.						
9	After 750 gm. meat.			0.853	0.859†	0.859†	0.859†
10	" 750 " "				1.11†	1.11†	1.11†
12	" 750 " "			0.951			
16	Glucose metabolism. 70 gm. glucose.	0.116					
17	Basal. Standard diet						
	Mar. 16.	0.094					
22	Basal. Standard diet						
	Mar. 31.	0.093					
30	4th day of fasting.	0.095					
Apr. 2	7th " " "	0.121					
10	15th " " "	0.082‡					
12	After ½ standard diet						
	Apr. 11.	0.104					
13	After ½ standard diet						
	Apr. 11 and 12.	0.81					
21	Basal.	0.120					
24	"	0.114					
27	2nd day of 750 gm. meat.					1.20	1.35
30	6th " " 750 " "				1.28	1.11	
May 1	7th " " 750 " "				1.23	1.24	
3	Day following meat.	0.317					

* Average of 2 hours.

† Dog in calorimeter. Average per hour.

‡ Average for 6 hours.

gm. per hr.

1st period (10.10—11.10) = 0.109
 2nd " (11.10— 2.16) = 0.083
 3rd " (2.16— 4.46) = 0.070

TABLE X.—*Respiration Experiments*

Date.	Experiment No.	Time.	CO ₂	O ₂	R. Q.	Urine N.	Non-protein R. Q.	Calories.	
								Protein.	
1917									
Jan. 5. . . .	2	2.00- 3.00	gm. 20.00	gm. 18.23	0.80	gm. (0.20)	0.80	5.30	
		3.00- 4.00	19.55	18.31	0.77	(0.20)	0.77	5.30	
" 6. . . .	3	11.00-12.00	23.79	17.96	0.96	(0.103)	0.97	2.73	
		12.00- 1.00	23.61	17.71	0.97	(0.103)	0.98	2.73	
" 8. . . .	4	12.00- 1.00	6.05	5.28	0.83	(0.20)	0.85	5.30	
" 11. . . .	6	1.30- 2.30	26.47	22.54	0.88	(1.00)	0.88	26.50	
		2.30- 3.30	27.85	24.93	0.81	(1.00)	0.82	26.50	

Sec									
Date.	Experiment No.	Time.	CO ₂	O ₂	R. Q.	Urine N.	Non-protein R. Q.	Calories.	
								Protein.	Alas
1917									
Feb. 28. . . .	8	11.00-12.00	gm. 23.69	gm. 21.07	0.82	gm. (0.2)	0.82	5.30	
		12.00- 1.00	22.88	20.73	0.80	(0.2)	0.80	5.30	
Mar. 1. . . .	9	11.00-12.00	8.18	5.75	1.03	0.103	1.07	2.73	
		12.00- 1.00	8.17	5.68	1.05	0.103	1.09	2.73	
" 2. . . .	10	10.30-11.30	7.13	5.52	0.94	0.108	0.97	2.86	
		11.30-12.30	6.70	5.14	0.95	0.108	0.98	2.86	
" 5. . . .	12	11.00-12.00	28.34	23.36	0.88	(0.103)	0.89	2.73	
		12.00- 1.00	26.97	23.04	0.85	(0.103)	0.85	2.73	
" 6. . . .	13	10.30-11.30	24.89	22.37	0.81	(0.103)	0.81	2.73	
		11.30-12.30	24.38	23.16	0.77	(0.103)	0.76	2.73	

* Standard diet at 5 p.m. day previous.

Dog XV.—First Series.

Calories.		Treadmill revolutions.	Work in meters traveled per hr.	Calories above the basal (17.5) per 1,000 meters traveled.	Energy in kg.-m. to move 1 kg. 1 meter.	Morning weight.	Food.
Non-protein.	Total calculated.						
55.59	60.89	8,610	3,925	11.0	0.585	8.0	No food and work.*
55.43	60.73	8,520					
59.94	62.67	8,617	3,936	11.1	0.579	8.35	Glucose, 70 gm. in water, 210 cc. at 9.55 a.m.* Dog refused water at end.
59.21	61.94	8,569					
12.22	17.52					8.3	Basal metabolism.* Average of 2 hrs.
48.36	74.86	8,756	4,010	14.3	0.657	9.25	Meat, 700 gm., at 9.45 a.m.*
55.64	82.14	8,955	4,101	15.8	0.724		

Series.

Calories.		Treadmill revolutions.	Work in meters traveled per hr.	Calories above the basal (17.2) per 1,000 meters traveled.	Energy in kg.-m. to move 1 kg. 1 meter.	Morning weight.	Food.
Non-protein.	Total calculated.						
65.47	70.77	9,382	4,303	12.3	0.580	9.0	No food.*
63.99	69.29	9,408					
17.35	20.08					9.3	Glucose, 70 gm., in 220 cc. water at 35° at 10 a.m.*
16.96	19.69						
16.16	19.02					9.15	Basal metabolism.*
14.87	17.73						
77.33	80.06	10,522	4,834	12.8	0.589	9.25	Glucose, 70 gm., in 210 cc. water at 35° at 10 a.m.* Vomited at 11.12 a.m.
75.47	78.20	10,590					
72.43	75.16	10,480	4,806	12.2	0.578	9.01	Work and no food.*
74.22	76.95	10,505					

TABLE I-
Second

Date.	Experiment No.	Time.	CO ₂	O ₂	R.Q.	Urine N.	Non-protein R.Q.	Calories.	
								Protein.	Alum.
1917									
Mar. 8 . . .	14	10.50-11.50	gm. 5.71	gm. 5.02	0.83	gm. 0.098	0.83	2.59	
		11.50-12.50	5.94	5.65	0.77	0.098	0.76	2.59	
" 9	15	1.30- 2.30	9.40	8.06	0.85	0.859	1.25	22.77	
		2.30- 3.30	9.68	8.55	0.82	0.859	0.93	22.77	
" 10	16	11.30-12.30	10.94	9.27	0.86	1.11	?	29.42	
		12.30- 1.30	10.58	9.64	0.80	1.11	0.85	29.42	
" 12	17	1.20- 2.20	31.40	27.54	0.83	0.951	0.84	25.21	
		2.20- 3.20	30.42	28.44	0.78	0.951	0.77	25.21	
" 13	18	10.00-11.00	23.22	22.32	0.76	(0.203)	0.75	5.38	
		11.00-12.00	23.59	22.98	0.75	(0.203)	0.75	5.38	
" 15	19	12.00- 1.00	28.55	22.22	0.94	(0.116)	0.94	3.07	
		1.00- 2.00	27.88	22.41	0.90	(0.116)	0.91	3.07	
" 16	20	11.00-11.30	4.18	2.69	1.13	0.058	1.20	3.07	
		11.30-12.30	8.49	5.79	1.07	0.116	1.12	3.07	
		12.30- 1.30	8.77	6.13	1.04	0.116	1.08	3.07	
" 17	21	10.00-11.00	5.55	4.67	0.86	0.094	0.88	2.49	
" 20	22	12.00- 1.00	27.15	25.32	0.80	(0.094)	0.77	2.49	10.14
		1.00- 2.00	25.90	24.06	0.78	(0.094)	0.78	2.49	6.17
" 22	23	12.00- 1.00	5.15	4.67	0.80	0.093	0.80	2.46	
" 23	24	12.00- 1.00	23.86	23.22	0.74	(0.093)	0.74	2.46	

* Standard diet at 5 p.m. day previous.

Continued.
Series.

Calories.		Treadmill revolutions.	Work in meters traveled per hr.	Calories above the basal (17.2) per 1,000 meters traveled.	Energy in kg.-m. to move 1 kg. 1 meter.	Morning weight.	Food.
Non- protein.	Total cal- culated.						
14.12	16.71					kg. 9.0	Basal metabolism.
16.02	18.61						
2.82	25.59					9.5	Meat, 750 gm., at 9.30 a.m.* 5th and 6th hours after meat.
4.48	27.25						
?	30.03					9.6	Meat, 750 gm., at 7.30 a.m.* 5th and 6th hours after meat.
0.61							
66.21	91.42	10,285	4,704	16.0	0.708	9.6	Meat, 750 gm., at 9.20 a.m. Work 5th and 6th hours after meat.
68.07	93.28	10,255					
68.41	73.79	10,230	4,688	12.2	0.585	8.9	Work and no food since meat.
70.42	75.80	10,235					
73.94	77.01	10,395	4,771	12.5	0.555	9.6	Glucose, 70 gm., in 210 cc. warm water at 10 a.m.*
74.06	77.13	10,440					
8.02	9.55					9.25	Glucose, 70 gm., in 210 cc. warm water at 9.30 a.m.* (Non-protein calories in- clude those in fat pro- duction.)
17.31	20.38						
18.44	21.51						
13.30	15.79					9.15	Basal metabolism.*
71.24	83.87	10,455	4,777	13.6	0.620	9.3	Alanine, 20 gm., + Lie- big's extract, 2 gm., + water, 150 cc., at 10 a.m.*
71.39	80.05	10,406					
13.07	15.53					9.25	Basal metabolism.*
74.25	76.71	10,300	4,717	12.6	0.582	9.20	Work without food.* Av- erage of 2 hrs.

TABLE I-
Second

Date.	Experiment No.	Time.	CO ₂	O ₂	R.Q.	Urine N.	Non-protein R.Q.	Caloria.	
								Protein.	Alkali.
1917									
Mar. 8. . . .	14	10.50-11.50	gm. 5.71	gm. 5.02	0.83	gm. 0.098	0.83	2.59	
		11.50-12.50	5.94	5.65	0.77	0.098	0.76	2.59	
" 9. . . .	15	1.30- 2.30	9.40	8.06	0.85	0.859	1.25	22.77	
		2.30- 3.30	9.68	8.55	0.82	0.859	0.93	22.77	
" 10. . . .	16	11.30-12.30	10.94	9.27	0.86	1.11	?	29.42	
		12.30- 1.30	10.58	9.64	0.80	1.11	0.85	29.42	
" 12. . . .	17	1.20- 2.20	31.40	27.54	0.83	0.951	0.84	25.21	
		2.20- 3.20	30.42	28.44	0.78	0.951	0.77	25.21	
" 13. . . .	18	10.00-11.00	23.22	22.32	0.76	(0.203)	0.75	5.38	
		11.00-12.00	23.59	22.98	0.75	(0.203)	0.75	5.38	
" 15. . . .	19	12.00- 1.00	28.55	22.22	0.94	(0.116)	0.94	3.07	
		1.00- 2.00	27.88	22.41	0.90	(0.116)	0.91	3.07	
" 16. . . .	20	11.00-11.30	4.18	2.69	1.13	0.058	1.20	3.07	
		11.30-12.30	8.49	5.79	1.07	0.116	1.12	3.07	
		12.30- 1.30	8.77	6.13	1.04	0.116	1.08	3.07	
" 17. . . .	21	10.00-11.00	5.55	4.67	0.86	0.094	0.88	2.49	
" 20. . . .	22	12.00- 1.00	27.15	25.32	0.80	(0.094)	0.77	2.49	10.14
		1.00- 2.00	25.90	24.06	0.78	(0.094)	0.78	2.49	6.17
" 22. . . .	23	12.00- 1.00	5.15	4.67	0.80	0.093	0.80	2.46	
" 23. . . .	24	12.00- 1.00	23.86	23.22	0.74	(0.093)	0.74	2.46	

* Standard diet at 5 p.m. day previous.

Continued.
Series.

Calories.		Treadmill revolutions.	Work in meters traveled per hr.	Calories above the basal per 1,000 meters traveled.	Energy in kg.-m. to move 1 kg. 1 meter.	Morning weight.	Food.
Non- protein.	Total cal- culated.						
7.45	20.05					kg. 9.4	Alanine. 20 gm., + Lie- big's extract, 2 gm., + water, 150 cc., at 9 a.m.*
13.41	22.04						
36.71	37.94	5,165				9.7	Glucose, 100 gm., in 250 cc. warm water at 10 a.m.*
72.49	74.95	10,370	4,737	12.5	0.550		
78.11	78.57	10,349					
72.51	74.97	10,276	4,718	12.6	0.570	9.40	No food.*
75.90	78.36	10,338					
71.15	73.66	10,475	4,796	12.0	0.584	8.75	Fasting. 3rd day, and work. Average of 2 hrs.
13.46	15.97					8.6	Fasting, 4th day, rest. Average of 2 hrs.
65.44	67.95	10,355	4,719	11.5	0.570	8.55	Fasting, 5th day, and work.
69.71	72.22	10,390					
11.75	14.95					8.35	Fasting, 7th day, rest. Average of 2 hrs.
12.53	14.70					8.10	Fasting, 8th day, rest.
11.71	13.88						
65.49	67.66	9,966	5,023	11.2	0.587		Fasting, 8th day, work.
67.59	69.76	10,885					On Apr. 3 and 4 at 5 p.m.
69.61	71.78	11,050					dog received $\frac{1}{2}$ portion of standard diet: meat, 50 gm.; biscuit meal, 50 gm.; lard, 10 gm.; bone ash, 10 gm.; calories 344 or 14.3 calories per hour.
12.24	14.41					8.20	Fasting, 9th day, rest. Average of 2 hrs.
66.30	68.47	10,434	4,777	11.3	0.587		Fasting (continued), 9th day. 2nd hr. of work.

TABLE I-
Second

Date.	Experiment No.	Time.	CO ₂	O ₂	R.Q.	Urine N.	Non-protein R.Q.	Calories.	
								Protein.	Alamin
1917			gm.	gm.		gm.			
Mar. 24 . . .	25	11.00-12.00	7.05	6.15	0.83	(0.093)	0.83	2.46	10.14
		12.00- 1.00	7.71	6.54	0.84	(0.093)	0.85	2.46	6.17
" 26 . . .	26	11.30-12.00	14.15	10.91	0.94	(0.093)	0.95	1.23	
		12.00- 1.00	28.45	21.45	0.96	(0.093)	0.97	2.46	
		1.00- 2.00	29.37	22.59	0.95	(0.093)	0.95	2.46	
" 27 . . .	27	11.00-12.00	25.18	22.19	0.83	(0.093)	0.83	2.46	
		12.00- 1.00	25.34	23.48	0.79	(0.093)	0.78	2.46	
" 29 . . .	28	12.00- 1.00	21.96	22.49	0.710	(0.095)	0.707	2.51	
" 30 . . .	29	12.00- 1.00	4.96	4.88	0.738	0.095	0.727	2.51	
" 31 . . .	30	11.00-12.00	20.93	20.66	0.737	(0.095)	0.734	2.51	
		12.00- 1.00	21.91	21.98	0.724	(0.095)	0.722	2.51	
Apr. 2 . . .	31	1.00- 2.00	4.66	4.59	0.738	0.121	0.719	3.20	
" 3 . . .	32	11.00-12.00	4.40	4.51	0.709	(0.082)	0.691	2.17	
		12.00- 1.00	4.19	4.26	0.716	(0.082)	0.697	2.17	
		1.00- 2.00	20.90	20.48	0.742	(0.082)	0.740	2.17	
		2.00- 3.00	21.25	21.14	0.730	(0.082)	0.727	2.17	
		3.00- 4.00	21.55	21.78	0.719	(0.082)	0.717	2.17	
" 5 . . .	33	11.00- 1.00	4.48	4.41	0.733	(0.082)	0.725	2.17	
		3.00- 4.00	20.89	20.78	0.731	(0.082)	0.728	2.17	

* Standard diet at 5 p.m. day previous.

ued.

n.	Calories.	Treadmill revolutions.	Work in meters traveled per hr.	Calories above the basal per 1,000 meters traveled.	Energy in kg.-m. to move 1 kg. 1 meter.	Morning weight.	Food.
	Total cal- culated.						
45	20.05					kg.	
41	22.04					9.4	Alanine, 20 gm., + Liebig's extract, 2 gm., + water, 150 cc., at 9 a.m.*
71	37.94	5,165				9.7	Glucose, 100 gm., in 250 cc. warm water at 10 a.m.*
49	74.95	10,370	4,737	12.5	0.550		
11	78.57	10,349					
51	74.97	10,276	4,718	12.6	0.570	9.40	No food.*
90	78.36	10,338					
15	73.66	10,475	4,796	12.0	0.584	8.75	Fasting, 3rd day, and work. Average of 2 hrs.
46	15.97					8.6	Fasting, 4th day, rest. Average of 2 hrs.
44	67.95	10,355	4,719	11.5	0.570	8.55	Fasting, 5th day, and work.
71	72.22	10,390					
75	14.95					8.35	Fasting, 7th day, rest. Average of 2 hrs.
53	14.70					8.10	Fasting, 8th day, rest.
71	13.88						
49	67.66	9,966	5,023	11.2	0.587		Fasting, 8th day, work.
59	69.76	10,885					On Apr. 3 and 4 at 5 p.m. dog received $\frac{1}{2}$ portion of standard diet: meat, 50 gm.; biscuit meal, 50 gm.; lard, 10 gm.; bone ash, 10 gm.; calories 344 or 14.3 calories per hour.
61	71.78	11,050					
24	14.41					8.20	Fasting, 9th day, rest. Average of 2 hrs.
30	68.47	10,434	4,777	11.3	0.587		Fasting (continued), 9th day. 2nd hr. of work.

TABLE I-
Secom

Date.	Experiment No.	Time.	CO ₂	O ₂	R.Q.	Urine N.	Non-protein R.Q.	Calories.	
								Protein.	Alamin.
1917									
Apr. 9 . . .	34	12.00- 1.00	gm. 3.88	gm. 3.78	0.746	gm. (0.082)	0.732	2.17	
		1.00- 2.00	18.64	18.71	0.726	(0.082)	0.721	2.17	
		2.00- 3.00	18.75	18.94	0.720	(0.082)	0.717	2.17	
		3.00- 4.00	19.21	19.49	0.717	(0.082)	0.713	2.17	
" 11 . . .	35	2.00- 3.00	3.92	3.85	0.739	(0.082)	0.725	2.17	
		3.00- 4.00	4.10	4.07	0.732	(0.082)	0.716	2.17	
" 13 . . .	36	11.00-12.00	4.14	3.80	0.80	0.081	0.79	2.14	
" 14 . . .	37	10.00-11.00	4.88	3.80	0.93	(0.104)	0.97	2.75	
" 21 . . .	40	11.00-12.00	4.93	4.09	0.88	0.120	0.90	3.18	
		12.00- 1.00	5.02	4.14	0.88	0.120	0.91	3.18	
" 24 . . .	41		4.87	3.96	0.90	0.114	0.92	3.02	
			5.13	4.31	0.87	0.114	0.88	3.02	
May 3 . . .	42	10.00-11.00	7.33	5.22	1.02	0.317	1.25	8.40	
		11.00-12.00	7.83	5.49	1.04	0.317	1.26	8.40	
		12.00- 1.00	7.08	5.19	0.99	0.317	1.19	8.40	
" 4 . . .	43	3.00- 4.00	11.22	9.53	0.86	(1.22)		32.39	
		4.00- 5.00	11.38	10.13	0.82	(1.22)		32.39	

*Concluded.
Series.*

Calories.		Treadmill revolutions.	Work in meters traveled per hr.	Calories above the basal per 1,000 meters traveled.	Energy in kg.-m. to move 1 kg. 1 meter.	Morning weight.	Food.
Non- protein.	Total cal- culated.						
10.20	12.37					kg. 7.60	Fasting (continued), 13th day, rest. Average of 2 hrs.
59.31	61.48	9,784	4,710	10.6	0.595		Same conditions, work.
60.07	62.24	10,384					
61.71	63.89	10,681					
10.43	12.60					7.45	Fasting (continued), 15th day, rest.
11.12	13.29						
10.46	12.60					7.60	½ portion of standard diet on Apr. 11 and 12 at 5 p.m. Average of 2 hrs.
10.24	12.99					7.90	Standard diet yesterday 5 p.m. Average of 2 hrs.
10.61	13.79					7.95	Basal. Standard diet yes- terday 5 p.m.
10.81	13.99						
10.39	13.41					8.05	Basal. Standard diet yes- terday 5 p.m.
11.49	14.51						
9.10	17.50					9.35	Basal. Apr. 25 to May 2, meat, 750 gm. at 10 a.m. and standard diet at 5 p.m.
10.15	18.55						
9.06	17.46						
(-1.85)	30.54					9.8	Meat, 750 gm. at 11 a.m. Standard diet yesterday at 5 p.m.
(-0.37)	32.02						

1

A METHOD FOR THE PREPARATION OF UNIFORM COLLODION MEMBRANES FOR DIALYSIS.

By CHESTER J. FARMER.

(From the Laboratory of Biological Chemistry, School of Medicine, Marquette University, Milwaukee.)

(Received for publication, October 23, 1917.)

The usefulness of collodion membranes for dialysis has been known for almost two decades. Various attempts have been made to control their permeability by varying the time of drying (1), by heating in water (2), by adding castor oil to the alcohol-ether solution of the guncotton (3), and by after-treatment of the thoroughly dried films with various strengths of alcohol (4).

The usual method of preparing these membranes consisted in forming them either on the outside or the inside of test-tubes. In the first case the test-tube is dipped into the collodion solution, allowed to drain a minute or two, then dried while being revolved in the air. When the membrane fails to stick to the finger, the tube is immersed in cold water for a few minutes, which causes the film to harden, and allows it subsequently to be peeled from the tube. The second method consists in filling the tube with collodion, then inverting it and allowing it to drain back into the stock bottle as completely as possible. The tube is then clamped in the vertical position (mouth downward) for a period varying from a few minutes to a half hour. This allows further drainage and also the slow evaporation of the solvent. When one fails to detect the odor of ether in the tube, it is filled with cold water which hardens the membrane and causes it to loosen from the wall of the tube. By inserting a small glass rod between the film and the tube, the membrane may readily be withdrawn. Membranes are always kept immersed in cold water until desired for use.

Membranes made as indicated above are lacking in uniformity, both as to thickness and dialyzing power. For this reason Walpole (5) discarded thimbles and prepared films by allowing a measured quantity of collodion solution containing a known amount of guncotton, to evaporate on a glass plate of definite area. The degree of drying was controlled by cutting from time to time, a small piece of film from the margin of the plate, immersing it in cold water for a few minutes, and then measuring its thickness by means of a micrometer. As the evaporation proceeded the thickness and permeability both decreased. When the desired thickness was attained, the plate was immersed in cold water until the film

had hardened sufficiently to allow it to be removed. In this way flat membranes were obtained with definite and uniform characteristics respective to strength and permeability. They could therefore be standardized and accurately described for purposes of duplication.

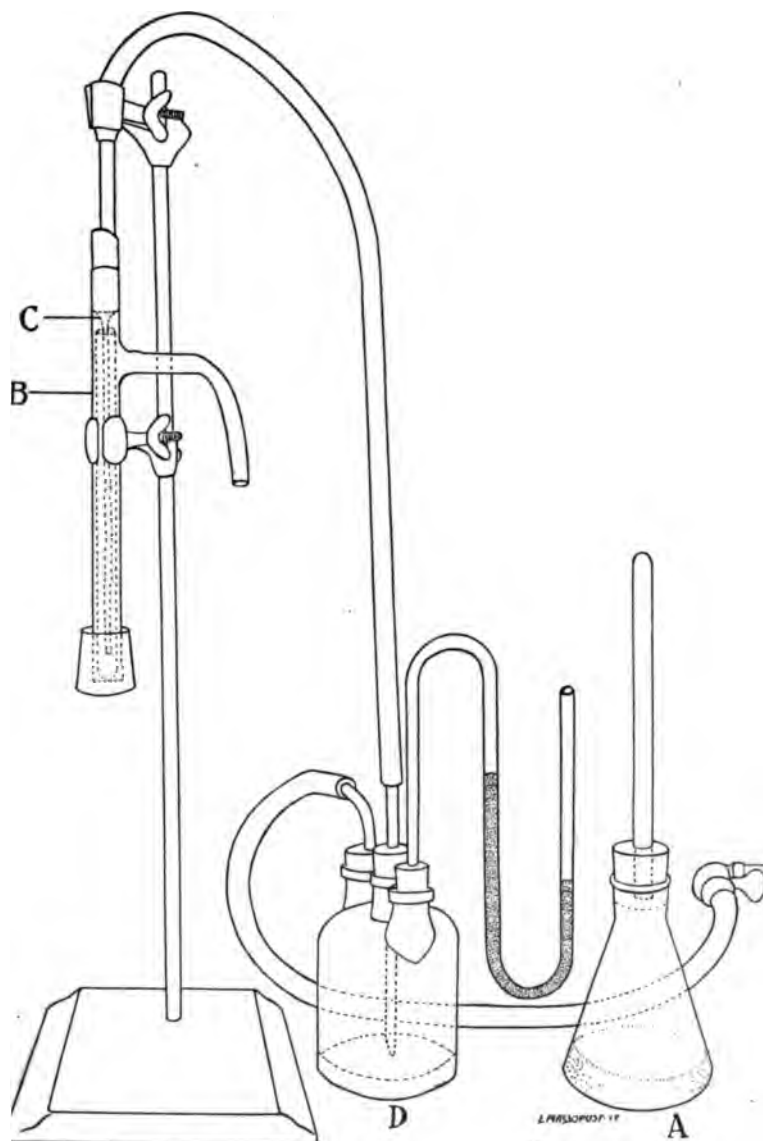
Recently Brown (4) has carefully studied the various factors entering into the preparation of graded dialyzing thimbles. His membranes are formed upon the outside of test-tubes. They are drained for 5 minutes into a half liter conical flask, being supported by the stopper. By thus limiting the evaporation a more uniform film of collodion is obtained. After drainage the tubes are immediately immersed in cold water for a minute. The membranes are then removed by eversion and allowed to become bone dry by exposure over night to the air. Such membranes are highly impermeable. The desired degree of permeability is obtained by subsequently soaking the thimbles for 24 hours in various strengths of alcohol; the greater the concentration of alcohol, the greater the permeability of the resulting membrane. After washing they are ready for standardization and use.

The purpose of this communication is to describe an apparatus for making collodion dialyzing membranes in which all variables except the time of drying are fixed. The apparatus has been in use in this laboratory for the last 2 years, and has given satisfaction in the preparation of thimbles ranging in diameter from 7 to 15 mm. Such membranes are easily duplicated, and may be standardized to various degrees of permeability.

Apparatus.

The apparatus is shown in the accompanying figure. It consists of a 150 cc. Erlenmeyer flask (A) into which is inserted a rubber stopper having a hole of sufficient size to allow easy insertion of the glass tube in which the membrane is to be formed. At the left (B) is shown the drying apparatus. As most of the membranes were 7 by 120 mm. in size, the measurements for this drier will be given. The jacket consists of a piece of tubing 155 mm. long and 9 mm. (inside) in diameter. At 25 mm. from the top a hole is blown, or preferably a tube of 8 mm. diameter sealed on to admit either laboratory air or air from a gas drying apparatus. Into the top of the jacket a piece of rubber tubing (about 25 mm. long) is inserted. Through this is pushed a glass tube of 5 mm. diameter which tapers uniformly from its point of emergence (C) to its tip where it has a diameter of 1 mm. The capillary should extend to within 5 or 6 mm. of the bottom of the

cket. It is important that the capillary should be concentric with the jacket, and parallel with its vertical axis. This adjust-



ment may easily be obtained by manipulating the rubber tubing through which the glass tube passes. The bottom of the jacket is closed by slipping over it a solid rubber stopper in which is bored a well extending to about two-thirds its depth. The top of the capillary (5 mm. tube) is connected by rubber tubing to the middle hole of a three-necked Wolff bottle (D). One of the end openings is connected with a vacuum pipe or water aspirator, while the other opening is plugged with a rubber stopper supporting an open end mercury manometer capable of recording 75 mm. of vacuum. The small tubes in which the membranes are formed are made by sealing off Jena or Pyrex tubing of 7 mm. bore into lengths of about 130 mm. The closed end is rounded like a test-tube. They slip readily to their entire length into the jacket of the drying apparatus.

Technique.

The collodion used for these membranes is prepared in the following way. 1 ounce of Anthony's negative cotton¹ is placed in a desiccator over sulfuric acid for 24 hours. 3 gm. of the dry material are placed in a 150 cc. Erlenmeyer flask, and 50 cc. of a mixture of equal parts of absolute alcohol and ether (distilled over sodium) are added. The flask is then stoppered and shaken occasionally. After about $\frac{1}{2}$ hour all evidence of fibrous material has disappeared, and the flask is allowed to stand quietly for another half hour to allow the air bubbles to collect and to come to the surface. After this the collodion solution is suitable for use. It has been recommended that the solution should be allowed to stand for 3 or 4 days in order that a brown sediment may settle out (6). This has not been found necessary if thoroughly dried guncotton and anhydrous ether and alcohol are used.

One of the small tubes (7 by 130 mm.) is pushed into the hole of the rubber stopper which fits the Erlenmeyer flask. It should not go completely through, but stop about 2 mm. before reaching the smaller end, as is shown in the figure. This is important for otherwise tubes of such small diameter will not drain con-

¹ Anthony's negative cotton is procurable in 1 ounce packages from the Ansco Company, Binghamton, N. Y.

pletely. Remove the stopper from the flask and insert the one carrying the tube. The flask is now inverted to an angle of 60° and the tube allowed to fill with collodion. Return the flask to an angle of 30° and allow the tube to drain, meanwhile revolving it about its axis. As soon as the column of collodion has drained out so that the tube seems empty, the flask is placed in the vertical position and allowed to drain *exactly 1 minute*. At the end of this time, quickly remove the tube and slip it vertically into the drying apparatus, closing the bottom of the jacket with the rubber stopper, as indicated in the diagram. After withdrawing the tube from the stopper of the Erlenmeyer flask, a glass plug is promptly inserted to prevent evaporation of the solvent. The suction should previously be started and adjusted so that the manometer records a vacuum of 50 mm. The tube is dried for *1 minute*, then immediately removed, and filled with cold distilled water. After a few minutes the collodion adhering to the mouth of the tube may be loosened by scraping with a pair of forceps. The free membrane is then pulled toward the opposite edge of the tube, permitting a little water to be forced out of the thimble and to run down between it and the glass. A small rod, 1 to 2 mm. in diameter, is inserted between the membrane and the tube wall, extending to the bottom. If the membrane has hardened sufficiently, it may be easily withdrawn by gentle traction on the forceps. Immerse the thimble in distilled water until desired for use.

Standardization.

The original object in making these membranes was to obtain dialyzing sacs suitable for use with the indicator method for determining the hydrogen ion concentration of blood or plasma. It was evident that the reliability of the method depended upon the uniform permeability of the membranes used, and that it would also be desirable to decrease the time of dialysis to a minimum.

To indicate the permeability, the technique of Levy, Rowntree, and Marriott (6) was adopted, substituting a Sørensen phosphate mixture of known pH value for blood. A series of ten thimbles was made, drying two of each respectively, 1, 2, 3, 4, and 5 minutes. After being thoroughly washed and tested for leaks with a physi-

ological salt solution, 3 cc. of phosphate mixture pH 8.2 were placed in each. They were then put into Jena test-tubes of approximately 9 by 100 mm. containing 3 cc. of physiological salt solution, which had been standing in a water bath at 40°C. for about 1 minute. After exactly 1 minute, all membranes were removed, the dialysate was allowed to cool to room temperature, and 0.3 cc. of a 0.01 per cent phenolsulfonephthalein solution added to each. They were then compared with the Sørensen standard tubes. The results are recorded in Table I.

TABLE I.
Membrane 7 by 120 Mm. Suction 50 Mm. Temperature 40°C.

Drain 1 min.; dry 1 min., pH 8.2 — 8.2
“ 1 “ “ 2 “ “ 8.0 — 8.0
“ 1 “ “ 3 “ “ 8.0 — 8.0
“ 1 “ “ 4 “ “ 7.9 — 7.9
“ 1 “ “ 5 “ “ 7.9 — 7.9

Standard phosphates used, pH 8.2.

Table I indicates that the permeability decreases with continued drying. As membranes dried but 1 minute offer no difficulty in being removed from the tube, it was decided to adopt this period as a standard for thimbles of this diameter. Membranes of larger diameters require a somewhat longer drying unless the volume of air is increased by using a higher vacuum.

In actual use the dialysis is conducted at room temperature rather than at 40°C. In order to determine the time required to establish equilibrium between the phosphate solution and the physiological salt solution, a series of nine thimbles was prepared and dialyzed for periods of 1, 2, and 3 minutes. Table II shows that 3 minutes was sufficient.

TABLE II.
Tubes 7 by 120 Mm. Suction 50 Mm.

	Dialysis.		
	1 min.	2 min.	3 min.
Drain 1 min.; dry 1 min.			
	7.5	7.7	7.8
Standard phosphate pH 7.8.	7.6	7.7	7.8
	7.8	7.7	7.8

The same result was obtained in the series indicated in Table III.

TABLE III.
Tubes 7 by 120 Mm. Suction 50 Mm.

	Dialysis. 3 min.
Drain 1 min.; dry 1 min.	
Standard phosphate, pH 8.0.	8.0
	8.0
	8.0

CONCLUSIONS.

An apparatus is described by means of which uniform collodion dialyzing membranes may be made. They are thinnest at the closed end, but owing to the opposed positions of the tube during drainage and drying the variations in the wall tend to be equalized.

The permeability and time of dialysis may be definitely established by standardization with phosphate mixtures. The small diameter of the thimble also favors rapid dialysis. The permeability may be changed by varying the period of drying. After establishing the proper drying time, any number of membranes having similar characteristics may be quickly prepared.

I wish to thank Mr. Leo Massopust for making the accompanying sketch of the apparatus.

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1

STUDIES OF ACIDOSIS.

VII. THE DETERMINATION OF β -HYDROXYBUTYRIC ACID, ACETO- ACETIC ACID, AND ACETONE IN URINE.*

By DONALD D. VAN SLYKE.

(From the Hospital of The Rockefeller Institute for Medical Research.)

(Received for publication, October 30, 1917.)

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*Reported at the Society for Experimental Biology and Medicine,
April 11, 1916.

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DESCRIPTION OF METHODS.

The methods are based on a combination of Shaffer's oxidation of β -hydroxybutyric acid to acetone and Denigès' precipitation of acetone as a basic mercuric sulfate compound. Oxidation and precipitation are carried out simultaneously in the same solution, so that the technique is simplified to boiling the mixture for an hour and a half under a reflux condenser, and weighing the precipitate which forms. The acetone and acetoacetic acid may be determined either with the β -hydroxybutyric acid or separately. Neither the size of sample nor mode of procedure have required variation for different urines; the same process may be used for the smallest significant amounts of acetone bodies and likewise for the largest that are encountered. The precipitate is crystalline and beautifully adapted to quick drying and accurate weighing; but when facilities for weighing are absent the precipitate can be redissolved in dilute hydrochloric acid and the mercury titrated with potassium iodide by the method of Personne (1863).

Preservatives other than toluene or copper sulfate should not be used.

Solutions Required.

20 per Cent Copper Sulfate.—200 gm. of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ dissolved in water and made up to 1 liter.

10 per Cent Mercuric Sulfate.—73 gm. of pure red mercuric oxide dissolved in 1 liter of H_2SO_4 of 4 N concentration.

50 Volume per Cent Sulfuric Acid.—500 cc. of sulfuric acid of 1.835 specific gravity, diluted to 1 liter with water. Concentration of H_2SO_4 must be readjusted if necessary to make it 17.0 N by titration.

10 per Cent Calcium Hydroxide Suspension.—Mix 100 gm. of Merck's fine light "reagent" $\text{Ca}(\text{OH})_2$ with 1 liter of water.

5 per Cent Potassium Dichromate.—50 gm. $\text{K}_2\text{Cr}_2\text{O}_7$ dissolved in water and made up to 1 liter.

Combined Reagents for Total Acetone Body Determination.—1 liter of the above 50 per cent sulfuric acid, 3.5 liters of the mercuric sulfate, 10 liters of water.

Removal of Glucose and Other Interfering Substances from Urine.

Place 25 cc. of urine in a 250 cc. measuring flask. Add 100 cc. of water, 50 cc. of copper sulfate solution, and mix. Then add 50 cc. of 10 per cent calcium hydroxide, shake, and test with litmus. If not alkaline, add more calcium hydroxide. Dilute to the mark and let stand at least one-half hour for glucose to precipitate. Filter through a dry folded filter. This procedure will remove up to 8 per cent of glucose. Urine containing more should be diluted enough to bring the glucose down to 8 per cent. The copper treatment is depended upon to remove interfering substances other than glucose, and should therefore *never be omitted, even when glucose is absent*. The filtrate may be tested for glucose by boiling a little in a test-tube. A precipitate of yellow cuprous oxide will be obtained if the removal has not been complete. A slight precipitate of white calcium salts always forms, but does not interfere with the detection of the yellow cuprous oxide.

Simultaneous Determination of Total Acetone Bodies (Acetone, Acetoacetic Acid, and Hydroxybutyric Acid) in One Operation.

Place in a 500 cc. Erlenmeyer flask 25 cc. of urine filtrate. Add 100 cc. of water, 10 cc. of 50 per cent sulfuric acid, and 35 cc. of the 10 per cent mercuric sulfate. Or in place of adding the water and reagents separately, add 145 cc. of the "combined reagents." Connect the flask with a reflux condenser having a straight condensing tube of 8 or 10 mm. diameter and heat to boiling. After boiling has begun, add 5 cc. of the 5 per cent dichromate through the condenser tube. Continue boiling gently $1\frac{1}{2}$ hours. (This time may, if desired, be shortened to 40 minutes by adopting the conditions described on page 474). The yellow precipitate which forms consists of the mercury sulfate-chromate compound (for

composition see p. 480) of the preformed acetone, and of the acetone which has been formed by decomposition of acetoacetic acid and by oxidation of the hydroxybutyric acid. It is collected in a Gooch or "medium density" alundum crucible, washed with 200 cc. of cold water, and dried for an hour at 110°. The crucible is allowed to cool in room air (a desiccator is unnecessary and undesirable) and weighed. Several precipitates may be collected, one above the other, without cleaning the crucible. As an alternative to weighing, the precipitate may be dissolved and titrated, as described below.

Acetone and Acetoacetic Acid.

The acetone plus the acetoacetic acid, which completely decomposes into acetone and CO₂ on heating, is determined without the hydroxybutyric acid exactly as the total acetone bodies, except that (1) no dichromate is added to oxidize the hydroxybutyric acid and (2) the boiling must continue for not less than 30 nor more than 45 minutes. Boiling for more than 45 minutes splits off a little acetone from hydroxybutyric acid even in the absence of chromic acid.

β-Hydroxybutyric Acid.

The hydroxybutyric acid alone is determined exactly as total acetone bodies except that the preformed acetone and that from the acetoacetic acid are first boiled off. To do this the 25 cc. of urine filtrate plus 100 cc. of water are treated with 2 cc. of the 50 per cent sulfuric acid and boiled in the open flask for 10 minutes. The volume of solution left in the flask is measured in a cylinder. The solution is returned to the flask, and the cylinder washed with enough water to replace that boiled off and restore the volume of the solution to 127 cc. Then 8 cc. of the 50 per cent sulfuric acid and 35 cc. of mercuric sulfate are added. The flask is connected under the condenser and the determination is continued as described for total acetone bodies.

Blank Determination of Precipitate from Substances in Urine Other than the Acetone Bodies.

The 25 cc. aliquot of urine filtrate is treated with sulfuric acid and water and boiled 10 minutes to drive off acetone. The resi-

due is made up to 175 cc. with the same amounts of mercuric sulfate and sulfuric acid used in the above determinations, but without chromate, and is boiled under the reflux for 45 minutes. Longer boiling splits off some acetone from β -hydroxybutyric acid, and must therefore be avoided. The weight of precipitate obtained may be subtracted from that obtained in the above determination.

The blank is so small that in our experience it is relatively significant only when compared with the small amounts of acetone bodies found in normal or nearly normal urines (see p. 488). In routine analyses of diabetic urines we do not determine it.

Test of Reagents.

When the complete total acetone bodies determination, including the preliminary copper sulfate treatment, is performed on a sample of distilled water instead of urine no precipitate whatever should be obtained. This test must not be omitted.

Titration of the Precipitate.

Instead of weighing the precipitate, one may wash the contents of the Gooch, including the asbestos, into a small beaker with as little water as possible, and add 15 cc. of 1 N HCl. The mixture is then heated, and the precipitate quickly dissolves. In case an alundum crucible is used, it is set into the beaker of acid until the precipitate dissolves, and then washed with suction, the washings being added to the beaker. In place of using either a Gooch or alundum crucible one may when titration is employed, wash the precipitate without suction on a small quantitative filter paper, which is transferred with precipitate to the beaker and broken up with a rod in 15 cc. of 1 N HCl.

In order to obtain a good end-point in the subsequent titration it is necessary to reduce the acidity of the solution. For this purpose we have found addition of excess sodium acetate the most satisfactory means. 6 to 7 cc. of 3 M acetate are added to the cooled solution of redissolved precipitate. Then the 0.2 M KI is run in rapidly from a burette with constant stirring. If more than a small amount of mercury is present, a red precipitate of

HgI_2 at once forms, and redissolves as soon as 2 or 3 cc. of KI in excess of the amount required to form the soluble K_2HgI_4 have been added. If only a few mg. of mercury are present, the excess of KI may be added before the HgI_2 has had time to precipitate, so that the titrated solution remains clear. In this case not less than 5 cc. of the 0.2 M KI are added, as it has been found that the final titration is not satisfactory if less is present. The excess of KI is titrated back by adding 0.05 M HgCl_2 from another burette until a permanent red precipitate forms. Since the reaction utilized is $\text{HgCl}_2 + 4 \text{KI} = \text{K}_2\text{HgI}_4 + 2 \text{KCl}$, 1 cc. of 0.05 M HgCl_2 is equivalent in the titration to 1 cc. of the 0.2 M KI.

In preparing the two standard solutions the 0.05 M HgCl_2 is standardized by the sulfide method, and the iodide is standardized by titration against it. A slight error appears to be introduced if the iodide solution is gravimetrically standardized and used for checking the mercury solution, instead of *vice versa*.

In standardizing the mercuric chloride we have found the following procedure convenient: 25 cc. of 0.05 M HgCl_2 are measured with a calibrated pipette, diluted to about 100 cc., and H_2S is run in until the black precipitate flocculates and leaves a clear solution. The HgS , collected in a Gooch crucible and dried at 110° , should weigh 0.2908 gm. if the solution is accurate.

Both by gravimetric analyses of the basic mercuric sulfate-acetone precipitate and by titration, we find the mercury content of the precipitate to average 76.9 per cent. On this basis, each cc. of 0.2 M KI solution, being equivalent to 10.0 mg. of Hg, is equivalent to $\frac{10.0}{0.769} = 13.0$ mg. of the mercury acetone precipitate.

Titration is not quite so accurate as weighing but, except when the amounts determined are very small, the titration is satisfactory (see p. 483).

Factors for Calculating Results.

1 mg. of β -hydroxybutyric acid yields 8.45 mg. of precipitate.

1 mg. of acetone yields 20.0 mg. of precipitate.

1 cc. of 0.2 M KI solution is equivalent to 13 mg. of precipitate in titration of the latter.

Special Factors for Calculation of Results when 25 Cc. of Urine Filtrate, Equivalent to 2.5 Cc. of Urine, Are Used for the Determination.

Determination performed.	Acetone bodies, calculated as gm. acetone per liter of urine, indicated by	
	1 gm. of precipitate. .	1 cc. of 0.2 M KI solution.
Total acetone bodies.*.....	24.8	0.322
β -hydroxybutyric acid.....	26.4	0.344
Acetone plus acetoacetic acid.....	20.0	0.260

* The "Total acetone bodies" factor is calculated on the assumption that the molecular proportion of them in the form β -hydroxybutyric acid is 75 per cent of the total, which proportion is usually approximated in acetonuria. (See for example, table on pp. 490-491). Because hydroxybutyric acid yields only 0.75 molecule of acetone, the factors are strictly accurate only when this proportion is present, but the error introduced by the use of the approximate factors is for ordinary purposes not serious. The actual errors in percentages of the amounts determined are as follows: molecular proportion of acetone bodies as β -acid 50, error -6.5 per cent; β -acid 0.60, error - 3.8 per cent; β -acid 0.80, error 1.3 per cent.

In order to calculate the acetone bodies as β -hydroxybutyric acid rather than acetone, use the above factors multiplied by the ratio of the molecular weights $\frac{\beta\text{-acid}}{\text{acetone}} = \frac{104}{58} = 1.793$. In order to calculate the acetone bodies in terms of molecular concentration, divide the factors in the table by 58. To calculate cc. of 0.1 M acetone bodies per liter of urine, use the above factors multiplied by $\frac{10,000}{58} = 172.4$.

DISCUSSION.

Since the cumulative results of Stadelmann (1883), Külz (1884), Minkowski (1884), and Magnus-Levy (1899) demonstrated the predominant rôle of β -hydroxybutyric acid in the production of diabetic coma,¹ need for satisfactory means of determining this sub-

¹ Stadelmann (1883) showed that an organic acid, yielding crotonic acid when distilled with strong sulfuric, is present in the urine in diabetic coma; Minkowski (1884) and Külz (1884) independently identified the organic acid as β -hydroxybutyric; and Magnus-Levy (1899) showed that the amounts present in the body after coma are sufficient to account for fatal acid intoxication. It has also been shown that when β -hydroxybutyric acid is formed

stance has been recognized. Of the resultant methods those which have proven practical are of two classes, polarimetric and oxidative. In the former the acid is extracted with ether, either directly from the acidified urine (for review of such methods see Hurtley, 1916) or from the powder obtained by mixing it with plaster of Paris (Black, 1908), and the acid, which has a specific rotation of -24.12° , is estimated by polarizing the extract. In the oxidative methods, which we owe to Shaffer, the hydroxybutyric acid is oxidized to acetone with sulfuric acid and dichromate. The acetone is distilled off during the oxidation and is collected and determined either iodometrically (Shaffer, 1908) or by determining the mercury precipitated by reaction of the acetone with Scott-Wilson's mercuric cyanide reagent (Marriott, 1913, *a*; Kennaway, 1914), or, for the minute amounts obtained in 1 cc. of blood, by estimating the Scott-Wilson precipitate with a nephelometer (Marriott, 1913, *b*; Folin and Denis, 1916).

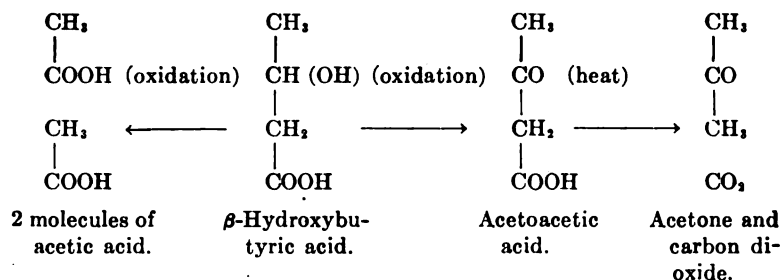
The extraction-polarimetric method is fairly reliable. The extraction, however, is time-consuming, and the polarization requires for determining the amounts of the substance found in urine the most accurate and expensive type of polariscope. Furthermore, only the β -hydroxybutyric acid is measured, not the acetoacetic acid nor the acetone, although these substances are no less significant of incomplete fat oxidation.

Presumably because of these disadvantages of the polarimetric methods, most of the determinations reported in recent years¹ have been made with the methods of oxidation and distillation. The Shaffer technique requires no expensive equipment, and as the acetone and the acetoacetic acid, which decomposes into

in the human organism it is accompanied by acetoacetic acid, usually in an amount not more than one-third that of the β -hydroxybutyric acid, as well as by a small amount of acetone, which is presumably formed by decomposition of the unstable acetoacetic acid. Both acids apparently occur in man whenever combustion of fatty acids derived from either fats or aminoacids is incomplete. The excreted β -hydroxybutyric and acetoacetic acids may amount to as much as 36 per cent of the consumed fat (Magnus-Levy, 1905).

¹ A noteworthy exception is the paper by Hurtley (1916) in which 48 hour extraction of the urine was used in routine analysis to obtain the hydroxybutyric acid, the acetone plus diacetic being determined by distillation.

acetone on boiling, can be obtained by a short preliminary distillation, all three "acetone bodies" can be determined in a single sample of urine, and with a single set of apparatus. The method has been, however, not entirely free from disadvantages. The acetone distilled from the oxidized hydroxybutyric acid is accompanied by impurities which necessitate treating with alkaline peroxide and redistilling before the acetone can be titrated. A more important drawback is the difficulty of maintaining the conditions necessary for constant results. As noted by Shaffer, about 10 per cent of the hydroxybutyric acid is, under the best conditions, decomposed by reactions which yield no acetone. Part of the oxidation breaks the molecule at the β -carbon, where the hydroxyl is attached, and yields other products than acetone, the chief among them being apparently acetic acid (see p. 474).



As a matter of fact, the oxidation may be so conducted that most of the product consists of substances which yield no acetone (see p. 473).

As shown by Shaffer, the yield of acetone is decreased by increase in concentration of chromic and sulfuric acids. On the other hand, the *speed* of oxidation increases with the concentration of these reagents (see pp. 469-470). When all concentrations are being constantly changed by removal of the water distilled away, and by reduction of chromic acid replaced according to the color changes of the mixture, it is difficult to avoid the occasional occurrence of either increased concentration of the reagents, with lowered acetone yield, or decreased concentration, with incomplete oxidation in the allotted time. Presumably because of the difficulty of controlling these factors, the Shaffer

determination makes unusual demands on the judgment of the analyst.

It seemed possible that the difficulties might be obviated by precipitating instead of distilling the acetone as it is formed. Denigès, in an investigation of organic compounds of mercury, found that acetone boiled under a reflux with a solution of sulfuric acid and mercuric sulfate forms a crystalline mercury complex approximately twenty times as heavy as the acetone, and of extraordinary insolubility both in dilute sulfuric acid and in water. He recommended it for precipitation of acetone directly in urine, and reported a few determinations. Since then Oppenheimer (1899) and Sammett (1913) have confirmed Denigès in tests on pure acetone solutions, and on normal urines to which acetone was added. Since Denigès' precipitant, like the chromic acid oxidizing agent, acts in sulfuric acid solution and at boiling temperature, it seemed possible to reduce the oxidative determination of hydroxybutyric acid to the simple procedure described in the first paragraph of this paper, eliminating both the labor of twice distilling the acetone and the errors attending fluctuation in the concentrations of the chromic and sulfuric acids. This expectation was realized only after a considerable amount of experimentation.

EXPERIMENTAL.

Removal of Glucose.

The first obstacle encountered was the necessity for complete removal of glucose. Amounts of it such as are encountered in diabetic urines invalidate the determination of either acetone or hydroxybutyric acid (see p. 486). This fact probably explains the failure of Denigès' method for acetone determination to attain use in urine analyses, since a large proportion of the urines in which it is desirable to determine acetone are from patients with diabetes mellitus.

The quantitative removal of glucose was attained by use of the fact, discovered by Salkowski (1879), that glucose forms a complex with copper which is completely precipitated when the solution contains a slight excess of alkali. We find that the optimum

alkalinity is attained by saturating the copper-glucose solution with calcium hydroxide. This not only causes complete precipitation of the glucose, but also precipitates all the excess copper as hydroxide; while of the lime itself, but little goes into solution. The precipitate when formed in urine also removes all of the colloidal and coloring matter, as well as some unidentified substances which if not removed form flocculent precipitates subsequently with the mercury reagent. The filtrate obtained from the copper precipitate is water-clear, and contains other than the three acetone bodies only ordinarily negligible traces of substances which yield mercury precipitates under the conditions of the determination. The amount of copper used by Salkowski to precipitate glucose was 5 molecules, one for each hydroxyl of the glucose. We find, however, that a little over half as much completely precipitates the sugar. Precipitation is not instantaneous, but requires 20 to 30 minutes.

The filtrate may be tested for glucose by merely boiling a few cc. in a test-tube. If any glucose remains in solution it holds some copper with it, and the latter is reduced when the filtrate is boiled. The test is not increased in sensitiveness by adding Fehling's or Benedict's copper solution. A slight white precipitate of calcium salts always forms, as previously mentioned, but this does not obscure the yellow of cuprous oxide when any sugar is present.

Experiment Showing Time Required for Complete Precipitation of Glucose.—In each of four 200 cc. flasks were placed 150 cc. of water, in which 1 gm. of Kahlbaum's glucose and 6 gm. of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (4.3 molecules) were dissolved. 3 gm. of pulverized calcium hydroxide were added; each mixture was shaken for a minute or two until it became alkaline to litmus, and then diluted to 200 cc. After varying intervals the solutions were passed through folded filters. The sugar in the filtrates was determined by polarizing in 2 dm. tubes. Since the polariscope could be read to $\pm 0.01^\circ$, it sufficed to determine any concentration of glucose exceeding 0.01 per cent. The filtrates were also tested for glucose qualitatively, both by Benedict's method and by merely boiling, as described above, without the addition of any reagent.

Interval between addition of lime and start of filtration.	Rotation of filtrate in 2 dm. tube.	Glucose in filtrate calculated from rotation.	Proportion of total glucose precipitated.	Benedict test for glucose in filtrate.	Reduction test by boiling filtrate alone.
min.	degrees	per cent	per cent		
1	+0.34	0.32	36	+++	+++
5	+0.15	0.14	72	++	++
15	+0.03	0.03	94	+	+
30	0.00	0.00	100	0	0
60	0.00	0.00	100	0	0

Experiment to Ascertain the Amount of Copper Sulfate Required to Precipitate 1 Gm. of Glucose.—This experiment was performed like the preceding, except that the copper sulfate was varied instead of the time allowed for precipitation, which was constant at 30 minutes. The calcium hydroxide used was proportional to the copper sulfate.

CuSO ₄ ·5H ₂ O		Calcium hydroxide.	Rotation of filtrate in 2 dm. tube.	Glucose unprecipitated.	Glucose precipitated.	Reduction test in filtrate.
gm.	mols. per 1 mol. glucose.	gm.	degrees	gm.	gm.	
2	1.44	1	+0.10	0.200	0.800	+++
4	2.88	2	+0.02	0.040	0.960	+
6	4.33	3	0.00	0.000	1.000	0
8	5.77	4	0.00	0.000	1.000	0

It is evident that 4.33 molecules of crystalline copper sulfate are sufficient to completely precipitate 1 molecule of glucose in half an hour. The fact that 1.44 molecules of copper sulfate precipitate approximately 80 per cent of the glucose indicates that the compound precipitated probably contains only 2 molecules of copper per molecule of glucose, rather than, as Salkowski thought, 1 molecule for each hydroxyl of the sugar.

Analysis and Rotation of Calcium-Zinc Hydroxybutyrate Used in Experiments.

The β -hydroxybutyric acid for all our experiments was weighed out in the form of the calcium-zinc salt from a recrystallized and analyzed preparation. The calcium-zinc salt was prepared by Dr. Vinograd-Villchur from diabetic urine by Shaffer and Marriott's method (1913) and recrystallized three times.

For analysis 0.500 gm. portions were dissolved in 10 cc. of water, and the calcium was precipitated as sulfate by the addition of 5 cc. of 2 N H_2SO_4 and 50 cc. of 95 per cent alcohol. The mixture was allowed to stand over night to precipitate. The calcium sulfate was collected in a Gooch crucible and ignited at a low red heat.

The filtrate was concentrated to a few cc. It was then diluted to 150 cc., neutralized with ammonia, and the zinc precipitated with 0.5 gm. ammonium phosphate. The precipitate was dried at 110° .

Substance.	CaSO_4	ZnNH_4PO_4	Ca	Zn
gm.	gm.	gm.	per cent	per cent
0.5000	0.1314	0.1708	7.72	12.52
0.5000	0.1318	0.1696	7.75	12.43
Calculated for $\text{CaZn}(\text{C}_4\text{H}_7\text{O}_2)_4$			7.74	12.62

Shaffer and Marriott's method for preparing the calcium-zinc salt yields such a beautifully crystalline product that the three recrystallizations were apparently superfluous. The first crop of crystals showed the same rotation as the last. The rotation of the first crop was taken on a solution containing 0.500 gm. of the salt in 20 cc. A 2 dm. tube with a Schmidt and Haensch spectropolarimeter was used.

$$[\alpha]_D^{20} = \frac{-0.77^\circ \times 20}{2 \times 0.500} = -15.4^\circ$$

The third crop gave the following figures: the solution used in this determination was twice as concentrated as the above.

$$[\alpha]_D^{20} = \frac{-1.54^\circ \times 20}{2 \times 1.000} = -15.4^\circ$$

To determine the rotation of the free acid, 0.1506 gm. of the salt, equivalent to 0.1210 gm. of free acid, was dissolved in 2 cc. of N HCl. The specific gravity of the solution was 1.040.

$$[\alpha]_D^{20} = \frac{-1.35^\circ \times 2.180}{0.1210 \times 1.040} = -23.37^\circ$$

Magnus-Levy (1899) gives -24.12 as the rotation of the pure acid, and Shaffer gives -16.2 as the rotation of the calcium-zinc salt. It is probable that our hydroxybutyric acid was slightly

racemized in the process of preparation. We have since found that a gradual loss of rotation occurs when the ether extract of the urine stands, and such extracts yield calcium-zinc salts of low rotation although they give perfect analytical figures for calcium and zinc, and likewise for β -hydroxybutyric acid determined as described in this paper.

Effect of Changes in Chromic and Sulfuric Acid Concentration.

Increase in either chromic or sulfuric acid concentration accelerates the oxidation, which by varying these concentrations can be made to run to completion in periods varied at will from 20 minutes to as many hours. Shortening of the reaction period by increasing sulfuric or chromic acid is attained, however, at the cost of a lowered acetone yield, since increase in either reagent increases the proportion of non-acetone products yielded by the oxidation. We are consequently forced to compromise between high yield and quick reaction time. Under the conditions upon which we finally settled as a routine, each molecule of β -hydroxybutyric acid yields 0.75 molecule of acetone, and the entire time required for both oxidation and precipitation of the mercury-acetone complex is 90 minutes. The conditions have been so studied, however, that any one may change them, completing the reaction in 40 minutes, for example, with 60 per cent yield. In the other direction one can, of course, increase the yield by diluting the chromate at will, so long as enough is present to complete the oxidation. The sulfuric acid, however, cannot be very much more dilute than under our standard conditions, or basic mercuric sulfate will be precipitated along with the acetone complex.

Experiment Showing Effect of Sulfuric Acid Concentration on the Velocity and Acetone Yield of the β -Hydroxybutyric Acid Oxidation.—Three sets of solutions were made up as follows. In each of a series of fifteen 500 cc. Erlenmeyer flasks were placed the following: 20 cc. of the 10 per cent HgSO_4 solution described at the beginning of this paper; 20 cc. of a solution containing per cc. 1.244 mg. of calcium-zinc hydroxybutyric, equivalent to 1 mg. of the free acid; an amount of 17 N sulfuric acid indicated in the table, and enough water to bring the total volume to 170 cc. The flasks were connected with reflux condensers and heated. After boiling had begun 5 cc. of 5 per cent $\text{K}_2\text{Cr}_2\text{O}_7$ solution were added to each solution through the reflux condenser. At varying time intervals measured from the moment when the dichromate was added the flasks were removed

and cooled quickly under tap water. The precipitates were washed and weighed as usual. The results are given in the following table and in Fig. 1.

17 N H ₂ SO ₄ added.	Concentration of H ₂ SO ₄ in total solution.	Weight of precipitate formed from 20 mg. of β -hydroxybutyric acid in time indicated.				
		30 min.	60 min.	90 min.	120 min.	150 min.
cc.	N	gm.	gm.	gm.	gm.	gm.
2	0.58	0.0982	0.1433	0.1676	0.1830	0.1884
8	1.17	0.1380	0.1650	0.1660	0.1708	0.1692
16	1.94	0.1314	0.1374	0.1356	0.1364	0.1354

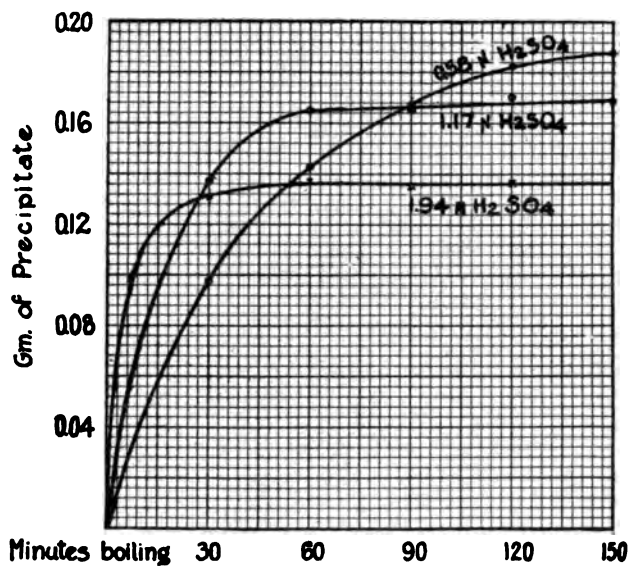


FIG. 1.

Experiment Showing the Effect of Chromic Acid Concentration on the Velocity and Acetone Yield of the β -Hydroxybutyric Acid Oxidation.—Each solution contained 10 mg. of β -hydroxybutyric acid. The conditions were those described at the beginning of this paper for determination of "Total acetone bodies" except for variations in the volume of 10 per cent chromate solution added. The volume of the total solution was in each case 175 cc. after the chromate solution had been added. The results are given in the following table and in Fig. 2.

$K_2Cr_2O_7$	Precipitate formed from 10 mg. β -hydroxybutyric acid after time indicated.				
	15 min.	30 min.	60 min.	90 min.	120 min.
gm.	gm.	gm.	gm.	gm.	gm.
0.15	0.0504	0.0752	0.0848	0.0884	0.0906
0.30	0.0652	0.0796	0.0846	0.0861	0.0865
1.00	0.0546	0.0662	0.0656	0.0660	—

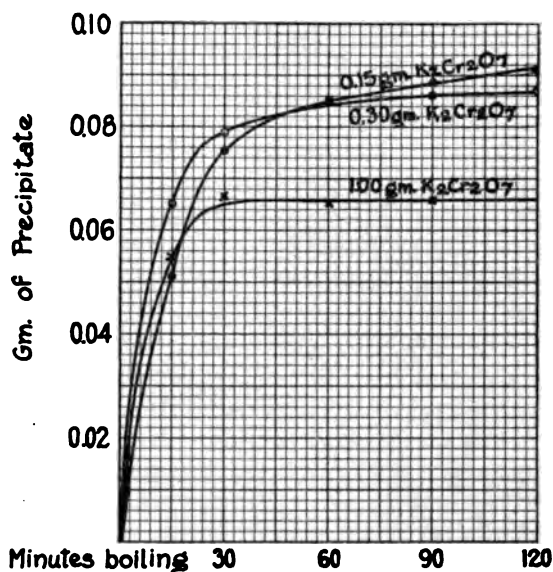


FIG. 2.

Effect of Mercuric Sulfate Concentration on Yield of Precipitate from β -Hydroxybutyric Acid.

Each flask contained 175 cc. of solution with 0.25 gm. of potassium dichromate and free sulfuric acid in sufficient amount to bring its concentration to 1.70 N, or 0.85 M (approximately 1 cc. of concentrated sulfuric to 20 cc. of solution). The mercuric sulfate was varied.

The precipitates obtained with concentrations of mercuric sulfate greater than 3 per cent were flocculent and abnormal in appearance. This was due to contamination with the yellow basic mercuric sulfate which is precipitated when mercury sulfate solutions are heated without sufficient free sulfuric acid to hold all

Concentration of HgSO_4 .		Yield of precipitate from 10 mg. β -hydroxybutyric acid.	
		After 1 hr. boiling.	After 2 hrs. boiling.
gm. per 100 cc.	gm. mols. per liter	gm.	gm.
1	0.034	0.0638	0.0674
2	0.068	0.0756	0.0772
3	0.101	0.0828	0.0832
4	0.133	0.0882	0.1036
5	0.169	0.0974	0.1492
6	0.203	0.1278	

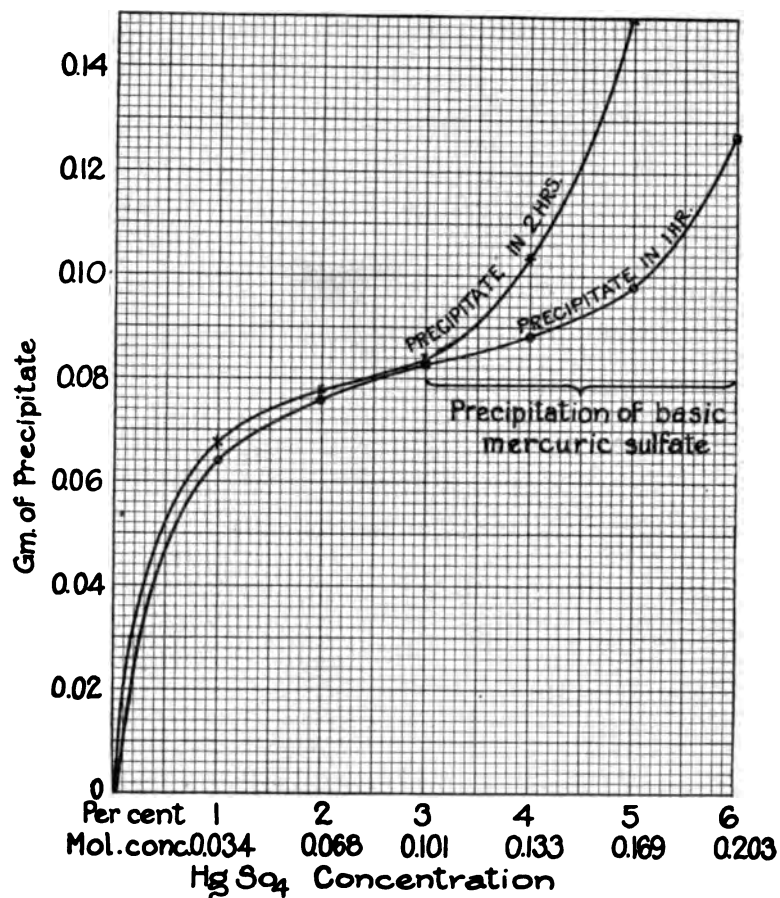


FIG. 3.

the mercury in solution. The point at which the precipitation of the basic salt begins is shown by the sharp change in direction of the curves of Fig. 3 as the 3 per cent point is passed. Up to this point the curves are concave, approaching a horizontal position at a level which evidently indicates the maximum yield of normally composed mercury-acetone precipitate. Beyond this point, however, the curves suddenly shoot upwards, in a manner explainable only by the formation of a new precipitate having no relation to the acetone present.

Control experiments with only sulfuric acid and mercury sulfate confirmed this explanation. With a greater $\frac{\text{HgSO}_4}{\text{H}_2\text{SO}_4}$ ratio than that prevailing at the 3 per cent experiment (approximately 1:8 in molecular proportions) precipitation of the basic mercuric sulfate occurs, with or without the presence of acetone. If the H_2SO_4 concentration is increased, the HgSO_4 can also be raised. The yield of precipitate from β -hydroxybutyric acid is diminished rather than increased, however, because of the previously discussed effect of sulfuric acid in diminishing the acetone yield. Thus, with 2.5 instead of 1.7 N H_2SO_4 the mercuric sulfate concentration can be raised to 5 per cent without precipitation of basic sulfate, but the yield of acetone precipitate is 10 per cent less than in the above experiment.

Influence of Temperature on the Yield of Acetone from β -Hydroxybutyric Acid.

In preliminary experiments to determine the yields of precipitate from varying amounts of β -acid it was found that in a given set of determinations run simultaneously with from 1 to 50 mg. of β -hydroxybutyric acid the yield of precipitate per mg. of acid was constant. Different sets of determinations run on different days, however, gave results which varied considerably, and we were for a time at a loss to explain this. The clue was finally given by two series, for both of which the solutions, including the dichromate, were mixed early one morning. One series was boiled in the morning and gave an average of 8.0 mg. of precipitate per mg. of acid. The other stood at room temperature until afternoon before it was boiled. In this series the yield of precipitate per mg. of hydroxybutyric acid was 7.2 mg. It appeared possible that in the

solution standing the longer time at room temperature a partial oxidation of the β -acid had occurred, and that at the low temperature the reaction took chiefly the path yielding products other than acetone. The following experiment shows that this explanation was correct.

The solutions were made up with reagents in the proportions described at the beginning of this paper for the routine method for "Total acetone bodies." The dichromate, however, was added *before* the solutions were heated, and allowed to act for varying periods at room temperature. The solutions were finally boiled under reflux for 1 hour, so that the intact β -acid would be oxidized in the usual manner. The amount of β -acid present was 18.04 mg. The following results were obtained:

Period of preliminary oxidation at room temperature.	Precipitate yielded on subsequent boiling for 1 hour.	
	From 18.04 mg. β -acid.	Per gm. β -acid.
	<i>gm.</i>	<i>gm.</i>
0	0.1480	8.21
(Dichromate added through reflux to boiling solution.)	0.1482	8.22
Dichromate added just before heating began.	0.1432	7.94
	0.1432	7.94
3 hours.	0.1274	7.06
	0.1270	7.04
20 "	0.0374	2.07
	0.0384	2.13

In 20 hours at room temperature three-fourths of the β -acid is destroyed by reactions which yield no acetone. Even when heating was begun immediately after the dichromate was added the portion of the oxidation that occurred before boiling temperature was reached reduced the acetone yield by 3 per cent. It was evident that low results must be encountered when any of the oxidation occurs at temperature below the boiling point. After this was discovered the routine precaution was introduced of adding the dichromate through the reflux condenser after boiling had begun, and the inconsistencies between different sets of determinations disappeared.

The Volatile Fatty Acid Formed by Oxidation of β -Hydroxybutyric Acid.

As pointed out on page 463, the structure of β -hydroxybutyric acid leads one to expect it to break partly in the middle on oxidation, yielding two molecules of acetic acid.

In order to obtain the volatile acid for analysis 0.5 gm. of the calcium-zinc salt was mixed with 170 cc. of water containing 20 cc. of the 50 volume per cent sulfuric acid and 20 cc. of 5 per cent potassium dichromate. The mixture was allowed to stand 48 hours in the cold. All the conditions, high sulfuric and chromic acid concentration and low temperature, tend to a low yield of acetone and high yield of the non-acetone products.

The solution was concentrated under diminished pressure to about 50 cc. and the distillate titrated with phenolphthalein as indicator. It was sulfate-free and neutralized 37.7 cc. of 0.1 N sodium hydroxide. The total β -hydroxybutyric acid present at the start would have neutralized 38.6 cc. The yield of volatile acid was therefore one molecule from one molecule of β -hydroxybutyric acid.

The distillate, after being neutralized by the titration, was concentrated to about 20 cc., poured into 80 cc. of absolute alcohol, and 0.5 gm. of silver nitrate in alcoholic solution was added. The white precipitate which formed was washed in a centrifuge with alcohol and ether. It weighed 0.35 gm., and gave the following figures on analysis.

0.3012 gm. of substance gave 0.2017 gm. Ag.

	Calculated for $\text{AgC}_2\text{H}_3\text{O}_2$:	Found:
Ag.....	64.7	66.9
	Calculated for AgCHO_2 :	
Ag.....	77.1	

The precipitate darkened quickly when exposed to light. This behavior and the analysis indicate that it was silver acetate, accompanied by a smaller amount of formate.

Experiment Illustrating Conditions for Determination of β -Hydroxybutyric Acid in 30 Minutes.

Oxidation and precipitation are accomplished most quickly with a high concentration of sulfuric acid and low mercuric sulfate. Solutions were made up as follows:

Volume.....	175 cc.
H ₂ SO ₄	1.91 N
HgSO ₄	2.00 gm. = 1.14 per cent
β -Hydroxybutyric acid.....	0.020 gm.
K ₂ Cr ₂ O ₇	0.250 gm.

The dichromate (5 cc. of 5 per cent solution) was added through the reflux condenser in each case after boiling had begun. The flask were removed from the flame at intervals and cooled under the tap. The yields of precipitate are shown below.

Time of boiling.	Yield of precipitate.	
	From 20 mg. β -acid.	Per gm. β -acid.
hrs.	gm.	gm.
0.5	0.1318	6.59
1.0	0.1310	6.55
1.5	0.1322	6.61
2.0	0.1318	6.59
3.0	0.1298	6.49

Both oxidation and precipitation were complete in 30 minutes. Since precipitation of acetone requires at least 15 minutes, it is evident that the oxidation itself must have been finished in about 15 minutes. The yield per gm. of β -acid, however, is only 6.6 gm., indicating the formation of but 0.60 molecule of acetone from each molecule of β -acid. Under the conditions chosen for routine work and described in the first part of this paper (2 per cent HgSO₄, 1.65 N H₂SO₄) 1½ hours of boiling are required, as shown below, to finish the precipitation, but the yield is 0.75 molecule of acetone.

Time Required for Complete Oxidation and Precipitation under Conditions Chosen for Routine Determination.

20 cc. portions of a solution containing 1.122 mg. of calcium-zinc hydroxybutyrate, or 0.903 mg. of hydroxybutyric acid, per cc. were treated in the manner prescribed for urine filtrate in "Determination of total acetone bodies" at the beginning of this paper. Only the period of boiling was varied as indicated in the following table.

Time of boiling.	Yield of precipitate.	
	From 18.06 mg. of β -hydroxybutyric acid.	Per gm. of β -hydroxybutyric acid.
<i>Ars.</i>	<i>gm.</i>	<i>gm.</i>
1	0.1478 0.1464	8.19 8.11
1.5	0.1518 0.1506	8.41 8.35
2.0	0.1512 0.1508	8.39 8.36

It is evident that both oxidation and precipitation are complete in 1.5 hours. In 1 hour the precipitate is 98 per cent of that obtained after 1.5 or more hours.

The Effect of Filtering Hot versus Filtering Cold.

Determinations were performed with solutions made up exactly as in the above experiment. The boiling was continued 1.5 hours in all cases. In one pair of determinations the precipitate was filtered immediately after the flame was turned out, while the solution was still boiling hot. In the other pair the solution was allowed to come to room temperature before it was filtered. In both cases cold wash water was used.

Yield of Precipitate from 18.12 Mg. of β -Hydroxybutyric Acid.

Filtered hot.	Filtered after cooling.
<i>gm.</i>	<i>gm.</i>
0.1494	0.1512
0.1502	0.1510
Average.....0.1498	0.1511

It appears that the hot liquid holds in solution about 1 mg. of mercury-acetone complex which adds itself to the precipitate when the mixture is cooled before filtering. The difference is so slight that it may in ordinary analyses be disregarded, and the precipitate filtered either hot or cold according to convenience.

Yield of Precipitate from Varying Amounts of β -Hydroxybutyric Acid.

Under the conditions chosen for routine determinations the yield of precipitate is approximately 8.45 gm. per 1 gm. of β -hydroxybutyric acid, and the ratio is not affected by any variations in the amount of β -acid present that are within the maximum amounts encountered in urine analyses.

β -hydroxybutyric acid.		Yield of precipitate.	
Present.	Corresponding to amount in urine.	Total.	Per 1 gm. β -hydroxybutyric acid.
gm.	per cent	gm.	gm.
0.001	0.04	0.0084	8.4
		0.0084	8.4
		0.0088	8.8
		0.0086	8.6
0.005	0.20	0.0422	8.44
		0.0424	8.48
0.010	0.40	0.0844	8.44
		0.0834	8.34
		0.0850	8.50
		0.0835	8.35
0.050	2.00	0.4336	8.42
		0.4364	8.45
		0.4396	8.49
		0.4374	8.47
0.100	4.00	0.9060	9.06
		0.8950	8.95

For amounts of β -hydroxybutyric acid equal to those which would be encountered in urines containing up to 2 per cent of the acid, the factor is constant at approximately 8.45 gm. of precipitate per gm. of acid. When 4 per cent of β -acid is present the factor is increased to 9.0; but since more than 2 per cent has, so far as we know, never been reported for a human urine, the factor 8.45 may be taken for all urine analyses.

With one mercury solution, made up from one of the cheaper

brands of mercuric sulfate, the β -hydroxybutyric acid factor was consistently lower, only 7.6 gm. of precipitate per 1 gm. of β -acid. The factor for pure acetone was not affected. Apparently the mercuric sulfate contained an unidentified impurity which affected the course of the oxidation. All the brands of red mercuric oxide which we were able to obtain gave consistent results, agreeing with those obtained with Merck's "reagent" mercuric sulfate, and consequently we have specified red mercuric oxide rather than mercuric sulfate in the directions for making up the mercury solution.

Time Required for Complete Precipitation of Acetone.

Solutions of acetone were treated as described for urine filtrates at the beginning of this paper, under "Determination of acetone and acetoacetic acid" except that the time of boiling was varied. The amount of acetone present was approximately 10 mg. (10 cc. of a 0.1 per cent solution made up by weight from Kahlbaum's "acetone from the bisulfite compound"). The flasks were cooled under the tap as quickly as possible after the period of boiling was over.

Time of boiling.	Yield of precipitate.
min.	gm.
5	0.1776
15	0.1940 0.1958
30	0.1962 0.1996
60	0.1960 0.1976

Yield of Precipitate from Varying Amounts of Acetone.

This experiment was conducted like the preceding, except that the time of boiling was kept constant at 30 minutes, the amounts of acetone being varied. Kahlbaum's "acetone from the

bisulfite compound" was dried with fused calcium chloride and redistilled, the first and last fractions being rejected and the middle fraction used for the following determination.

0.9670 gm. of this acetone was weighed out into a stoppered flask containing 50 cc. of water. After mixing, the solution thus obtained was diluted to 2 liters, so that 1 cc. contained 0.4835 mg. of acetone. 20 cc. of this solution were diluted to 200, and portions of the diluted solution used for the determinations of acetone amounts below 2 mg. The determinations were carried out as described for "Acetone and acetoacetic acid" at the beginning of this paper.

Yields of Precipitate from Varying Amounts of Acetone.

Acetone.	Precipitate.	Average wt. of precipitate.	Precipitate per 1 mg. acetone.
<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
0.242	4.4 4.8	4.6	19.0
0.484	9.6 9.6	9.6	19.8
0.967	20.0 19.6	19.8	20.5
1.934	40.4 40.0	40.2	20.7
2.901	57.6 58.6	58.1	20.0
3.868	78.2 78.0	78.1	20.2
4.835	96.0 94.8	95.4	19.74
9.670	189.8 191.2	190.5	19.70
19.31	377.5 377.9	377.7	19.55

Under the condition for the determination of acetone and acetoacetic acid the yield of precipitate is, within the limits of

analytical error, 20 mg. It falls slightly below 20 with the larger amounts, but for urine and blood analysis, where 150 mg. is the maximum precipitate from acetone and acetoacetic acid, the factor 20 may be taken throughout without causing significant error.

Composition of the Precipitates Obtained from β -Hydroxybutyric Acid, and from Acetone in Absence and Presence of Chromic Acid.

50 cc. portions of solutions containing approximately 1 mg. of β -hydroxybutyric acid or 0.5 mg. of acetone per cc. were precipitated as described, for the determinations in urine filtrates, of β -hydroxybutyric acid, total acetone bodies, and acetoacetic acid. The precipitates were weighed in Gooch crucibles, then transferred with the asbestos to beakers, where they were dissolved in 20 cc. portions of normal hydrochloric acid. The asbestos was filtered out, the washings diluting the solutions to about 100 cc. The mercury was precipitated as sulfide and dried to constant weight at 110° in Gooch crucibles. The filtrate from the sulfide was boiled free of H_2S , and the SO_4 was precipitated by slow addition of 10 cc. of 5 per cent barium chloride to the hot solution. The BaSO_4 was ignited and weighed in Gooch crucibles. The filtrate from the barium sulfate was freed from barium by addition of sulfuric acid, and any chromate which might have escaped reduction by H_2S was reduced by boiling with alcohol. The chromium was then precipitated as hydroxide with ammonia, was ignited to Cr_2O_3 , and weighed in platinum crucibles. The results are given in the accompanying table.

It is evident that, when chromic acid is present in the concentrations used, mercuric chromate or dichromate replaces about one-fourth the mercuric sulfate in the precipitate. The mercury content of the precipitate is but little affected, ranging from 76.6 to 77.0 per cent, whether or not chromate is present and whether the precipitate arises from preformed acetone or from acetone from hydroxybutyric acid.

The composition in the absence of chromate is most nearly indicated by the formula $3\text{HgSO}_4 \cdot 5\text{HgO} \cdot 2(\text{CH}_3)_2\text{CO}$, which agrees closely with the observed figures for SO_4 and Hg but indicates 5.5 per cent of acetone instead of the 5.0 per cent (shown by the 1:20 ratio of acetone to precipitate) which is the maximum that can be present if all the acetone is precipitated and none destroyed. Denigès (1898) gave $2\text{HgSO}_4 \cdot 3\text{HgO} \cdot (\text{CH}_3)_2\text{CO}$, which, however, indicates 14.8 per cent SO_4 , instead of the 13.71 to 13.97 which

we find, and only 4.6 per cent of acetone. It appears that the composition varies somewhat according to the conditions under which the precipitate is formed.

Composition of Precipitates.

	Precipitates from 50 cc. of β -hydroxybutyric acid solution boiled with chromic acid as in determination of β -hydroxybutyric acid.			Precipitates from 50 cc. acetone solution boiled in presence of chromic acid, as in determination of total acetone bodies.			Precipitates from 50 cc. acetone solution boiled without chromic acid, as in determination of acetone plus acetoacetic acid.		
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Mercury-acetone precipitate....	0.4532	0.4528	0.4510	0.4884	0.4858	0.4870	0.4776	0.4766	0.4742
HgS.....	0.4052	0.4044	0.4010	0.4316	0.4316	0.4373	0.4246	0.4232	0.4198
BaSO ₄	0.1184	0.1186	0.1178	0.1184	0.1184	0.1186	0.1621	0.1598	0.1600
Cr ₂ O ₃	0.0178	0.0186	0.0175	0.0215	0.0217	—			
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Hg.....	77.10	77.01	76.60	76.10	76.58	76.58	76.60	76.58	76.30
SO ₄	10.76	10.78	10.75	9.97	10.02	9.94	13.97	13.71	13.88
CrO ₄	5.98	6.27	5.92	6.78	6.81	—	0	0	0

Molecular Proportions.

	Average of three determinations.		
Hg.....	8.00	8.00	8.00
SO ₄	2.35	2.18	3.02
CrO ₄	1.09	1.23	0
CrO ₄ + SO ₄ ..	3.44	3.41	3.02
Cr ₂ O ₇ + SO ₄ ...	2.90	2.80	3.02

When chromate enters the composition it is possible, because of the well known tendency of CrO₄ salts to form isomorphous crystals with SO₄ salts, that the chromium is in the form CrO₄ rather than Cr₂O₇. It is impossible to decide the point from the analytical figures, however. If the Cr is calculated as CrO₄, the combining power of CrO₄ + SO₄ is greater (3.4 molecules) than that of SO₄ alone (3.0 molecules) when no chromate is present.

The absolute amount of Hg, as well as of the total precipitate yielded by a given amount of acetone, is also increased by the presence of chromate (average precipitate = 0.487 gm. with chromate, 0.476 without; HgS yields being 0.431 and 0.422 gm. respectively from these precipitates). It therefore appears that the HgCrO₄

or HgCr_2O_7 ; not only replaces about one-fourth of the HgSO_4 in the acetone precipitate, but is also added onto the precipitate sufficient to increase by about 1 part in 40 the weight of the precipitate yielded by a given amount of acetone.

As the result of this fact it would be logical in the calculation of the results of "Total acetone body" determinations, in which chromate is used, to employ the factor 20.5 rather than 20 in estimating the results. It is a matter of empirical observation, however, that when acetone and β -hydroxybutyric acid are thus determined together, there seems to be a slight compensating error, so that the precipitate does not exceed by more than the limit of experimental error the sum of the precipitates obtained in determination of the two substances separately (see table on p. 490). We have therefore used the factor 20 throughout for calculating acetone.

Titration of Mercury in the Precipitates.

The mercury-acetone precipitate can be readily dissolved in warm dilute hydrochloric acid and estimated by titration of the mercury, since, as shown above, the mercury content is approximately constant at 76.6 to 77.0 per cent. Denigès (1898) himself titrated by adding to the mercuric salt solution an excess of 0.1 N KCN, and titrating with 0.1 N AgNO_3 the cyanide in excess of the amount required to form $\text{Hg}(\text{CN})_2$. Sammett (1913) did not get satisfactory results with the method. We have tried the Volhard titration of the mercury with sulfocyanate. Although it gave excellent results with pure mercuric sulfate solution, it was not even approximately accurate with the redissolved acetone precipitate. We have, however, like Willaman (1916), obtained good results with the old method of Personne (1863).

The following results obtained with the technique already described on pages 459-460, indicate the degree of accuracy of the titration.

Effect on β -Hydroxybutyric Acid of the Reagents Used in Determining Acetone and Acetoacetic Acid.

Even without chromic acid, β -hydroxybutyric acid when subjected to prolonged boiling with sulfuric acid and mercuric sulfate

splits off a little acetone and yields a weighable precipitate. The amount formed from pure β -acid however is not measurable if the time of boiling is kept below 45 minutes. Nor does presence

Results Obtained by Titration of the Mercury in the Acetone Precipitates.

β -hydroxybutyric acid.	Wt. of precipitate.	0.2 M KI.	0.05 M HgCl ₂ .	Excess 0.2 M KI.	β -hydroxybutyric acid calculated from titration (excess KI \times 1.538).	Amount of precipitate calculated from titration (excess KI \times 13.0).
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Standard solutions of calcium-zinc salt of β -hydroxybutyric acid.

mg.	mg.	cc.	cc.	cc.	mg.	mg.
2	—	5.00	3.70	1.30	2.00	—
2	—	5.00	3.65	1.35	2.07	—
2	—	5.00	3.70	1.30	2.00	—
10	86.2	8.00	1.45	6.55	10.07	85.2
10	86.0	8.00	1.50	6.50	10.00	84.5
20	171.4	15.00	2.00	13.00	20.00	169.0
20	169.2	15.00	2.25	12.75	19.61	164.8
30	—	21.00	1.15	19.85	30.53	—
30	—	21.00	1.30	19.70	30.24	—
50	—	35.00	1.90	33.10	50.91	—
50	—	35.00	1.75	33.25	51.13	—

Precipitates from total acetone body determinations in urines.

—	69.6	8.00	2.85	5.15	—	66.9
—	67.6	8.00	2.90	5.10	—	66.3
—	70.8	8.00	2.75	5.25	—	68.3
—	138.0	12.50	2.35	10.15	—	132.0
—	140.4	12.50	1.80	10.70	—	139.1
—	678.0	55.00	2.90	52.10	—	677.5
—	645.0	55.00	5.40	49.70	—	646.0

of the β -acid appreciably affect the results of the acetone determination if in the latter the period of boiling is kept under 45 minutes.

Experiment.—250 cc. of water, 0.500 gm. of calcium-zinc hydroxybutyrate, 100 cc. of the 20 per cent $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ solution, and 10 gm. of calcium hydroxide were made up to 500 cc. and filtered, as in the preparation of urine for analysis. Of the filtrate 25 cc. portions were treated in Nos. 1 and 2 as described at the beginning of this paper for "Acetone plus acetoacetic acid" and " β -hydroxybutyric acid" determinations, the only variation in

technique being in the duration of the boiling period for the acetone plus acetoacetic acid determination. In Nos. 3 and 4, 10 cc. portions of 0.102 per cent acetone were added, while 5 and 6 serve as control analyses of the acetone alone.

No.	Acetone present.	β -acid present.	Yield of precipitate.					
			Acetone determination (no dichromate).				β -hydroxybutyric acid determination (boiled 90 min. with dichromate).	
			Boiled 45 min.		Boiled 90 min.		Precipitate.	β -acid calculated from precipitate.
			Precipitate.	Acetone calculated from precipitate.	Precipitate.	Acetone calculated from precipitate.		
	mg.	mg.	gm.	mg.	gm.	mg.	gm.	mg.
1	0	20.1	0.0000	0	0.0032	1.62	0.1684	19.9
2	0	20.1	0.0002	0	0.0048	2.43	0.1720	20.3
3	10.2	20.1	0.1998	9.99				
4	10.2	20.1	0.2036	10.18				
5	10.2	0	0.2022	10.11				
6	10.2	0	0.2012	10.06				

Determination of β -Hydroxybutyric Acid by Heating at 100° in Pressure Bottles. (Alternative to Boiling under Reflux).

Oppenheimer (1899) and Sammett (1913) found that acetone could be determined by heating with the mercury reagent in a pressure bottle immersed in a water bath as well as by Denigès' original method of boiling under a reflux. β -hydroxybutyric acid also may be determined by carrying out the oxidation and precipitation in a pressure bottle, and this technique may be used as an alternative to boiling under a reflux. Because of the lower temperature of oxidation, a greater part of the β -acid is converted into products other than acetone, so that the yield of precipitate is only three-fourths as great as when the reflux is used. The results also are less constant, but it appears that when reflux apparatus is not available pressure bottles may be used if a high degree of accuracy is unnecessary. The following experiment shows that precipitation is completed about as quickly in the pressure bottles as under the reflux.

Portions of 10 mg. of β -hydroxybutyric acid were placed in 400 cc. bottles

with patent clamps (ordinary magnesium citrate bottles) with the reagents, including dichromate in the amounts previously prescribed for the determination of β -hydroxybutyric acid. The bottles were closed and nearly covered with cold water in a large kettle. The water was heated to boiling as quickly as possible. At stated intervals, measured from the moment when boiling began, bottles were removed, and the precipitates which had formed were collected and weighed in Gooch crucibles.

Time.	Precipitate.	
	Weighed.	Per gm. β -acid.
<i>hrs.</i>	<i>gm.</i>	<i>gm.</i>
0.5	0.0372	3.72
1.0	0.0576	5.76
1.5	0.0666	6.66
2.0	0.0646	6.46
2.5	0.0664	6.64
3.0	0.0668	6.68
3.5	0.0646	6.46
4.0	0.0648	6.48

The following yields were obtained from varying amounts of β -hydroxybutyric acid, the time of heating at 100° being uniform at 2 hours.

β -hydroxybutyric acid present.	Precipitate.	Precipitate per gm. β -hydroxybutyric acid.
<i>mg.</i>	<i>mg.</i>	<i>gm.</i>
2	14.8	7.4
	14.8	7.4
10	61.8	6.18
	60.4	6.04
50	346.2	6.92
	346.6	6.93

It is evident that although good duplicate determinations with a given amount of β -hydroxybutyric acid are obtained, the yield of precipitate per gm. of the acid varies much more with different amounts of the acid than when the reflux condenser is used. In case it is necessary to use the pressure bottle, 6.5 gm. of precipitate per gm. of β -hydroxybutyric acid may be taken as the average yield, but variations of 10 per cent in the results must be expected,

unless the pressure bottle technique is worked out in more detail than it has been as presented here.

Effect of Other Organic Substances on the Results of the β -Hydroxybutyric Acid Determination.

Routine β -hydroxybutyric acid determinations were performed on solutions containing 10 mg. of β -hydroxybutyric acid with and without the addition of other organic substances which either occur naturally in urine or might be added to it as antiseptics.

Added to 10 mg. β -hydroxybutyric acid.			Yield of precipitate.	Effect on precipitate of added substance.	
Substance.	Amount.	Corresponding to concentration in urine sample.		Total effect.	Effect per 1 mg. added substance.
	mg.	per cent	mg.	mg.	mg.
0	0	0	84.8	—	—
0	0	0	84.8	—	—
Lactic acid.....	10.0	0.4	91.4	+6.6	+0.7
“ “	50.0	2.0	161.6	+76.8	+1.3
Urea.....	50.0	2.0	84.6	0	0
Creatine.....	5.0	0.2	89.2	+4.4	+0.9
Uric acid.....	5.0	0.2	86.8	+2.0	+0.4
Glucose.....	20.0	0.8	80.8	-4.0	-0.2
“	100.0	4.0	65.8	-19.8	-0.2
“ *	200.0	8.0	841.0	+756.2	+3.8
“ **	500.0	20.0	233.2	+2251.4	+4.5
Thymol.....	To saturate 2.5 cc.	Saturated solution.	107.0	+22.2	—
Phenol.....	2.5	0.1	101.8	17.0	+6.8
“	10.0	0.4	133.2	48.4	+4.8
Ethyl alcohol....	2.5	0.1	80.6	-4.2	-1.7
“ “	10.0	0.4	118.6	+33.8	+3.2
“ “	100.0	4.0	1004.6	+919.8	+9.2

* Required 0.6 gm. $K_2Cr_2O_7$.

** Required 0.9 gm. $K_2Cr_2O_7$.

Urea in the amounts ordinarily present in urine has no effect on the yield of precipitate. Creatine (which would be converted

at least partially into creatinine by the boiling in acid), lactic acid, and uric acid give precipitates, but not to such an extent that the amounts ordinarily encountered in urines would significantly affect the results obtained in ketonuria. Glucose has a peculiar effect. Amounts less than 0.1 gm. apparently interfere with the oxidative formation of acetone, as they reduce the yield of precipitate from the β -hydroxybutyric acid. Amounts over 0.2 gm., corresponding to 8 per cent glucose in a urine sample, yield enormous precipitates. If a solution of pure glucose is boiled with the reagents to determine acetone or hydroxybutyric acid, the solution may remain clear for 20 to 30 minutes but eventually a precipitate begins to form and increases rapidly. It is evident that glucose must be removed before the determination is performed, and we have consequently introduced its precipitation by copper into the routine technique.

The effect of alcohol is like that of glucose; small amounts decrease the yield of precipitate, larger amounts enormously increase it, doubtless from the formation of acetaldehyde. As only 2 mg. of alcohol appreciably affect the results, care must be taken that none of the flasks or pipettes used in the analysis are wet with it.

Neither thymol nor phenol (nor of course formaldehyde) may be used as preservative for urines which are to be used for these determinations.

Analyses of Normal Urines.

The following analyses show the range of results for total acetone bodies that may be obtained with normal urines. The urea plus ammonia nitrogen is given as an indication of the concentration of the urine.

The maximum is 0.28 gm. per liter calculated as acetone or 0.50 gm. per liter calculated as β -hydroxybutyric acid. If no corrections were made for the blanks, the largest precipitate obtained, 16.8 mg., would indicate 0.42 gm. per liter calculated as acetone, or 0.75 gm. per liter calculated as β -hydroxybutyric acid. While peculiar diets could doubtless cause higher figures, these may be taken as the maxima likely to be encountered in normal men under usual conditions.

Subject.	Precipitates from 25 cc. filtrate, equivalent to 2.5 cc. urine.			Total acetone bodies in urine calculated as acetone.	Urea plus ammonia nitrogen.
	Total acetone bodies precipitate uncorrected.	Blank precipitate.	Total acetone bodies precipitate minus blank precipitate.		
	mg.	mg.	mg.	gm. per liter	gm. per liter
1. V. S.....	3.0	1.4	1.6	0.04	5.77
2. G. E. C.....	1.0	2.8	1.8	0.04	9.45
3. J. A. P.....	2.0	2.1	0.1	0.00	7.28
4. C. L.....	5.0	4.3	0.7	0.02	0.24
5. F. B.....	5.8	4.5	1.3	0.03	9.57
6. W. H.....	5.0	3.2	1.8	0.04	0.80
7. R. F.....	9.8	3.8	6.0	0.15	4.82
8. W. T.....	11.7	5.6	6.1	0.15	9.92
9. H. M.....	10.6	5.4	5.2	0.13	1.20
10. E. S.....	3.2	2.9	0.3	0.01	1.87
11. F. K.....	5.5	2.4	3.1	0.08	2.15
12. B. S.....	4.8	3.6	1.2	0.03	2.77
13. W. W. P.....	11.8	8.2	3.6	0.09	—
14. A. M. L.....	16.0	5.0	11.0	0.27	1.87
15. E. T.....	12.2	5.4	6.8	0.17	—
16. R. F.....	15.2	4.0	11.2	0.28	—
17. W. T.....	16.8	6.0	10.8	0.27	—
18. A. S.....	15.2	6.4	8.8	0.22	—
19. R. J. N.....	10.6	9.0	1.6	0.04	—
20. D. O.....	6.8	2.6	4.2	0.10	7.61
21. W. J.....	8.8	4.4	4.4	0.11	9.07
22. S. L.....	14.0	4.2	9.8	0.24	8.81
23. H. K.....	5.0	2.0	3.0	0.07	5.32

The maximum blank determination yielded 9 mg. of precipitate equivalent to 0.22 gm. per liter of acetone, or 0.40 gm. per liter of hydroxybutyric acid.

We have also performed a considerable number of blank determinations on diabetic urine. The blanks average about the same as in normal urines (see table on p. 490).

Analysis of Urine to Which Glucose, β -Hydroxybutyric Acid, and Acetone Were Added.

3 gm. of glucose, 0.195 gm. of acetone, and 0.3182 gm. of β -hydroxybutyric acid (0.3952 gm. of calcium-zinc salt) were dissolved in 50 cc. of normal urine. The urine solution was poured into a 500 cc. flask, diluted with 200 cc. of water, and treated with 100 cc. of 20 per cent copper sulfate solution plus an excess of calcium hydroxide in the usual manner. 25 cc. portions of the glucose-free filtrate, equivalent to 2.5 cc. of the urine, were used for determinations performed as described at the beginning of the paper.

Acetone plus Acetoacetic Acid Determination.

Precipitate from urine alone.	Precipitate from urine plus added substances.	Precipitate from added substances.	Acetone calculated from precipitate.	Acetone present.
gm.	gm.	gm.	per cent	per cent
0.0014	0.1932 0.1928	0.1918 0.1914	0.383 0.383	0.390

β -Hydroxybutyric Acid Determination.

Precipitate from urine alone.	Precipitate from urine plus added substances.	Precipitate from added substances.	β -hydroxybutyric acid calculated from precipitate.	β -hydroxybutyric acid present.
gm.	gm.	gm.	per cent	per cent
0.0028	0.1380 0.1372	0.1352 0.1344	0.640 0.638	0.6364

Total Acetone Bodies.

Precipitate from urine alone.	Precipitate from urine plus added substances.	Precipitate from added substances.	Precipitate calculated as acetone \times 0.0200 plus β -acid \times 0.00845.
gm.	gm.	gm.	gm.
0.0040	0.3280 0.3284	0.3240 0.3244	0.3295

Analyses of Diabetic Urines. Comparisons of Results with Those by Black's Extraction-Polarization Method.

The data in the following table show the nature of the results obtained in conditions varying from normal to diabetic ketonuria with

TABLE.

Date.	Urine excreted in 24 hrs.	Blank determination.		β -hydroxybutyric acid determination.		Acetone + acetoacetic acid determination.		Total acetone bodies determination.			Total acetone bodies calculated as sum of average separate β -hydroxybutyric acid and acetone + acetoacetic determinations. Calculated as				Molecular proportion of acetone bodies in form of β -acid.	β -hydroxybutyric acid determined by Black's polarimetric method.																																																																																																																																																																																																																																																																																																											
		Pre-cipitate corrected.	β -hydroxybutyric acid per liter urine calculated as	Pre-cipitate corrected.	Acetone + acetoacetic acid per liter urine calculated as	Pre-cipitate corrected.	Total acetone bodies per liter urine calculated as	Pre-cipitate corrected.	Total acetone bodies per liter urine calculated as	Pre-cipitate corrected.	Total acetone bodies per liter urine calculated as	Pre-cipitate corrected.	Total acetone bodies per liter urine calculated as																																																																																																																																																																																																																																																																																																														
														gm.	cc. 0.1 M		gm. β -hydroxybutyric acid	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M	gm. β -hydroxybutyric acid	cc. 0.1 M

25	3375	0.0018	0.2662	1212	12.59	7.03	0.0944	326	3.39	1.89	0.3574	1525	15.90	8.85	16.15	54.5	1.18	78.9	12.6	99
		0.0028	0.2720	1238	12.86	7.18	0.0948	327	3.40	1.90	0.3546	1514	15.77	8.78					10.4	82
26	4090	0.0017	0.2318	1154	10.96	6.12	0.0691	238	2.48	1.38	0.3050	1302	13.57	7.57	14.62	59.8	1.30	83.2		
		0.0021	0.2382	1184	11.26	6.19	0.0681	235	2.44	1.36	0.3060	1306	13.61	7.59						
27	4430	0.0030	0.1843	839	8.72	4.86	0.0773	267	2.77	1.55	0.2568	1096	11.42	6.37	11.52	51.0	1.11	76.0		
		0.0012	0.1850	842	8.24	4.88	0.0769	265	2.76	1.54	0.2566	1095	11.41	6.36						
28	5610	0.0000	0.0614	279	2.91	1.62	0.0377	130	1.36	0.75	0.0994	424	4.42	2.46	4.23	23.8	0.52	68.1	2.7	93
		0.0000	0.0606	276	2.87	1.60	0.0378	130	1.36	0.76	0.0998	426	4.44	2.47						
29	5080	0.0004	0.0322	146	1.52	0.85	0.0155	52	0.56	0.31	0.0474	202	2.11	1.17	2.10	10.6	0.23	72.9		
		0.0004	0.0324	147	1.53	0.85	0.0170	57	0.61	0.34	0.0472	202	2.10	1.17						
Mar.																				
1	5080	0.0006	0.0216	98	1.02	0.57	0.0113	38	0.41	0.23	0.0327	140	1.45	0.81	1.41	7.2	0.16	74.3	0.8	75
		0.0008	0.0226	103	1.07	0.60	0.0093	32	0.33	0.19	0.0332	137	1.43	0.80				0.6	66	
3	3450	0.0006	0.0138	63	0.65	0.36	0.0105	35	0.38	0.21	0.0268	114	1.19	0.67	1.05	3.6	0.08	64.5	0.7	108
		0.0004	0.0136	62	0.64	0.36	0.0099	34	0.36	0.20	0.0266	113	1.18	0.66				0.8	123	
6	3450	0.0004	0.0028	13	0.13	0.07	0.0016	5	0.06	0.03	0.0040	17	0.18	0.10	0.20	0.7	0.01	63.2	0.4	330
		0.0008	0.0024	11	0.11	0.06	0.0024	8	0.09	0.05	0.0056	24	0.25	0.14						

a nearly maximal concentration and output of acetone bodies per kilo of body weight. The figures are from a patient whose alkaline reserve curves, diet, etc., are given in Chart 6, page 424, of Paper VI. All the data on this case taken together illustrate the course of an acidosis and ketonuria, both initially intense, and both successfully treated by a low calorie meat diet gradually decreased to a complete fast. It may be noted that the patient's clinical condition followed the alkaline reserve rather than the ketonuria. The patient, at first with acute gastric, respiratory, and nervous signs of impending coma, became free from these symptoms as soon as the alkaline reserve approached normality, although the ketonuria continued at the same high level for several days longer. The patient was under the care of Dr. Edgar Stillman.

Blank determinations of the amounts of precipitate yielded by substances other than the acetone bodies were performed in duplicate on each urine as previously described. The results are recorded in the third column of the table, and the "corrected" weights given for the other precipitates in the case of each urine are those actually obtained diminished by the mean weight of the blank. The amounts of precipitate obtained in the blank determinations are, however, so small in proportion to the amounts obtained in the β -hydroxybutyric and total acetone body determinations during ketonuria that they are for practical purposes unimportant.

In the last column of the table are given for comparison the results of determinations on some of the same urines performed by Dr. Vinograd-Villchur with Black's method. The procedure as described by Black (1908) was followed without deviation except that the concentrated urines were made acid to Congo instead of to litmus, and the extraction was continued 6 hours instead of 2. It was found that unless the more acid end-point of Congo was used there was danger of only partially freeing the β -hydroxybutyric acid from its salts. Black's procedure is simple, and, as is seen, yielded from 85 to 100 per cent of the β -hydroxybutyric acid determined by our gravimetric method. That the polarimetric results are usually somewhat low is not surprising, since the known possible errors, *viz.*, incomplete extraction, the slight racemization or destruction during extraction mentioned

by Shaffer and Marriott, and adsorption of the acid by charcoal used in clearing the final solution, all are such as to lower the results.³

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³ Freundlich (1906) has shown that charcoal partially adsorbs the lower fatty acids, formic, acetic, propionic, and butyric from water solution. Some adsorption of hydroxybutyric acid therefore seems probable.

STUDIES OF ACIDOSIS.

VIII. THE DETERMINATION OF β -HYDROXYBUTYRIC ACID, ACETO-ACETIC ACID, AND ACETONE IN BLOOD.

By DONALD D. VAN SLYKE AND REGINALD FITZ.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

(Received for publication, October 30, 1917.)

The technique for determining the acetone bodies in urine has been ascertained to be directly applicable to blood after a proper method had been found for removing the proteins from the latter.¹ Satisfactory results were obtained by precipitating the proteins at room temperature with the same mercuric sulfate solution (73 gm. of red mercuric oxide dissolved in 1 liter of 4 N H₂SO₄) utilized for precipitation of the acetone. The mercury-protein precipitate leaves no interfering substances in solution, and it absorbs none of the acetone bodies: both β -hydroxybutyric acid and acetone added to blood are quantitatively recovered by the process described below.

Whole Blood.—Of whole blood 10 cc. are diluted with about 100 cc. of water in a 250 cc. flask, and 20 cc. of the 10 per cent mercuric sulfate are added. The solution is shaken for a moment, until the protein coagulates, and is then diluted with water up to the 250 cc. mark. After 15 minutes or longer it is filtered through a dry folded filter. If the first drops are cloudy they are passed through a second time. The filtrate has a slight pink tinge, but the substance responsible for it does not precipitate when boiled with mercuric sulfate, nor otherwise interfere with any of the acetone body determinations.

If the blood is diluted with much more than ten volumes of water before the mercury is added, coagulation of the proteins is considerably slower,—hence the reason for not completing the dilution until after the coagulation has occurred.

¹ Colloidal ferric hydroxide 6 cc. added cold per 1 cc. of whole blood, gives a beautiful protein-free filtrate, but the precipitate absorbs about one-third of the β -hydroxybutyric acid present

Plasma or Serum.—8 cc. of oxalate plasma or of serum are diluted in a 200 cc. flask with 50 cc. of water and 15 cc. of the mercuric sulfate are added. The flask is shaken for a moment, until the fine precipitate which first forms has flocculated, and is then filled to the mark with water. After standing 15 minutes or longer the solution is filtered.

Determinations.—For determination of acetone plus acetoacetic acid, of β -hydroxybutyric acid, or of the total acetone bodies together, 125 cc. of the filtrate, equivalent to 5 cc. of either blood or plasma, may be treated exactly as the 25 cc. of urine filtrate plus 100 cc. of water in urine analyses.

In case, however, it is desired to *determine separately the acetone plus acetoacetic acid and the hydroxybutyric acid in a single sample of blood*, this may be done by first precipitating the preformed acetone plus that from acetoacetic acid, and then determining the hydroxybutyric acid in the filtrate. The preformed acetone plus that from acetoacetic acid is precipitated exactly as in urine analysis. The filtrate from the mercury-acetone precipitate is received into a dry flask. After as much of the solution as possible has been filtered through, and before any wash water has been used, 160 cc. of the filtrate, equivalent to $\frac{4}{5} \times 5$ cc. of blood, are placed in a 500 cc. Erlenmeyer flask, heated to boiling under a reflux condenser, and 5 cc. of 5 per cent potassium dichromate solution are added through the condenser. The rest of the hydroxybutyric acid determination is carried out as described for urine from the point where the dichromate is added.

Factors for Calculating Results When Filtrate Equivalent to 5 Cc. of Blood Is Used for Determination.

Determination performed.	Acetone bodies, calculated as gm. of acetone per liter of blood, indicated by	
	1 gm. of precipitate.	1 cc. of 0.2 M KI solution.
Total acetone bodies.....	12.8	0.161
β -hydroxybutyric acid.....	13.2 (14.0)*	0.172 (0.183)*
Acetone plus acetoacetic acid.....	10.0	0.130

* These factors are used when β -hydroxybutyric acid is determined in the filtrate from the precipitated acetone and acetoacetic acid as described above. In this case the amount of filtrate taken for the β -acid determination is equivalent to only $\frac{4}{5}$ of 5 cc. of blood, and the factor must be correspondingly increased.

To calculate the acetone bodies as β -hydroxybutyric acid instead of as acetone, multiply the above factors by 1.793; to calculate molecular concentration, divide the factors by 58.

Normal blood when analyzed as described for total acetone bodies yields only 1 or 2 mg. of precipitate, equivalent to 0.013 to 0.026 gm. of acetone per liter. In diabetics as much as 2.5 gm. of acetone bodies calculated as acetone has been observed, while patients under ordinarily good control show 0.1 to 0.4 gm.²

² Several hundred determinations of β -hydroxybutyric acid and acetone plus acetoacetic acid have been performed by Dr. Fitz on the blood of diabetic patients in a study of the conditions influencing the formation of the different acetone bodies, and of the effect of the latter on the blood bicarbonate. Publication of the results is delayed by Fitz' sudden call to military duty while engaged in the problem.—D. D. V. S.

STUDIES OF ACIDOSIS.

IX. RELATIONSHIP BETWEEN ALKALI RETENTION AND ALKALI RESERVE IN NORMAL AND PATHOLOGICAL INDIVIDUALS.

By WALTER W. PALMER AND DONALD D. VAN SLYKE.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

(Received for publication, October 30, 1917.)

Sellards (1912) and Palmer and Henderson (1913) have shown that in normal individuals the administration of 5 or 10 gm. of sodium bicarbonate is sufficient to turn the urine alkaline; while in patients suffering from acidosis a greater amount is required. Palmer reports patients with uremia who received 112 gm. of bicarbonate and still excreted acid urine.

These results suggest that the kidneys secrete alkaline urine only when the bicarbonate concentration of the blood exceeds a certain level. To judge from the small amounts of bicarbonate required to turn the urine of normal men alkaline, this level would be appreciably, but not greatly above the average normal.

The work reported in the present paper was undertaken to ascertain:

1. Whether a definite level of the plasma bicarbonate does exist in normal adults, at which neutral urine is excreted, while higher bicarbonate levels cause an alkaline, and lower an acid urine. (For the sake of brevity we will term the plasma bicarbonate level at which neutral urine is excreted the *critical level*.)

2. Whether in case such a definite level exists, it is altered by disease; *i.e.*, whether in a nephritic, for example, the blood bicarbonate must be raised to a higher level than in a normal man before the urine becomes alkaline.

3. Whether absorbed or injected bicarbonate is so distributed through the body that the body weight being known, the extent to which a given dose of bicarbonate will raise the blood bicarbonate may be calculated.

That solutions of these questions have practical application as

well as theoretical interest is obvious. The bicarbonate retention test of Palmer and Henderson, and Sellards is probably the simplest of all methods for the detection and approximate measurement of acidosis, and it is essential that its results should be compared with those of direct measurements of the plasma bicarbonate. The solution of (1) is necessary in order that the results of the retention test in acidosis may be intelligently interpreted in terms of internal alkali deficit; of (2) in order that the diseases, if such exist, may be known in which the retention test, because of a shift in the critical level, gives abnormal results, since in such conditions it would be unsafe to use the reaction of the urine as an indicator of the internal alkaline reserve; of (3) in order that when an alkali deficit in the blood plasma has been measured the therapeutic dose of bicarbonate required to make good the alkali deficit of the body may be estimated.

EXPERIMENTAL.

Sodium bicarbonate in 2 gm. amounts in 100 cc. of water was given by mouth every half hour to normal and pathological subjects until the alkalinity of the urine reached that of normal blood, a pH of approximately 7.4. In certain pathological cases, after four or five doses, if no appreciable effect on the reaction of the urine was noted the dose was increased to 5 gm. The hydrogen ion concentration of the urine was estimated before alkali was started and at half hourly intervals thereafter; and at the time of the administration of the alkali. Blood bicarbonate determinations were made just before alkali was given and again at the point when the reaction of the urine reached a pH of 7.4.

For the determination of the pH of the urine the colorimetric method described by Henderson and Palmer (1913) was employed. Plasma bicarbonate estimations were made as described by Van Slyke and Cullen.

If the sodium bicarbonate is distributed uniformly to all the body fluids we can calculate approximately the rise in blood bicarbonate caused by the absorption of 1 gm. of the salt. The calculation, in terms of plasma CO_2 , is made as follows: 1 gm. of NaHCO_3 contains 267 cc. of CO_2 measured at 0° , 760 mm. If the body fluids are estimated at 700 cc. for each kilo of body weight

then the distribution of 1 gm. of bicarbonate among them would raise the CO_2 content, in cc. per 100 cc. of fluid by $\frac{267}{7W} = \frac{38}{W}$ cc., W representing the body weight in kilos. If g gm. of bicarbonate were taken into the fluids, the rise in volume per cent of CO_2 would be $\frac{38g}{W}$. Conversely, the amount of bicarbonate necessary to raise the CO_2 by b volume per cent would be $g = \frac{bW}{38}$. If this equation holds even approximately, the fact shows that absorbed bicarbonate attains a fairly uniform distribution throughout the body.

We have separated our results into two groups, Table I containing the normal and Table II the pathological subjects. The several individuals are arranged in order of the number of gm. of alkali necessary to reduce the urinary reaction to that of normal blood, a pH of 7.4.

Two of the pathological cases in Table II, Experiments 17 and 19, became nauseated before the pH of the urine reached 7.4, hence the experiment was discontinued.

TABLE I.
Normal Subjects.

Experiment No.	Subject.	Weight. kg.	pH of urine.		Combined CO_2 in plasma.		Amount of NaHCO_3 given. gm.	Calculated increase in CO_2 , $\frac{38g}{W}$.	Observed increase in CO_2 .	Difference between calculated and observed CO_2 increase.
			Before NaHCO_3 .	After NaHCO_3 .	Before NaHCO_3 .	After NaHCO_3 .				
					vol. per cent	vol. per cent				
1	R. T.	66	6.9	7.4	66.2	68.1	2	1.2	1.9	-0.7
2	C. L.	86	5.4	7.3	64.5	67.7	4	1.8	3.2	-1.4
3	E. S.	90	5.3	7.4	63.2	65.6	4	1.7	2.4	-0.7
4	W. W. P.	90	7.0	7.5	71.4	70.9	6	2.5	-0.5	+3.0
5	D. D. V. S.	75	7.2	7.4	68.6	74.9	6	3.0	6.3	-3.3
6	W. W. P.	90	7.2	7.4	65.5	69.8	6	2.5	4.3	-1.8
7	R. F.	70	6.2	7.5	70.7	72.8	8	4.3	1.4	+2.9
8	F. G. B.	65	5.7	7.4	69.1	75.7	10	5.9	6.6	-0.7
9	W. W. P.*	90	6.1	8.1	68.5	72.8	20	8.4	4.3	+4.1

* The 20 gm. of NaHCO_3 were taken at once. Second sample of blood $1\frac{1}{2}$ hours later.

TABLE II.
Pathological Subjects.

Experiment No.	Case No.	Weight.		pH of urine.		Combined CO ₂ in plasma.		Time between taking first and second blood samples.	Amount NaHCO ₃ given.	Calculated increase in CO ₂ , 38g. W.		Observed increase in CO ₂ .	Difference between calculated and observed CO ₂ increase.	Remarks.		
		kg.		Before NaHCO ₃	After NaHCO ₃	Before NaHCO ₃	After NaHCO ₃			vol. per cent	vol. per cent				vol. per cent	vol. per cent
				vol. per cent	vol. per cent	hrs.	gm.									
10	2988	26	5.5	7.4	62.8	73.9	1½	8	11.7	11.1	+0.6	Diabetes without ketonuria.				
11	2867	64	5.1	7.4	69.5	75.8	1½	8	4.8	6.3	-1.5	Pleurisy with effusion.				
12	2793	80	7.0	7.4	76.6	77.5	3	12	5.7	0.9	+4.8	Chronic nephritis; cardiac decompensation.				
13	2907	60	5.0	7.4	68.0	77.7	3	15	9.5	9.7	-0.2	Chronic nephritis.				
14	3046	43	6.6	7.4	67.0	74.0	4	16	14.1	7.0	+7.1	Diabetes with ketonuria.				
15	2805	65	7.0	7.4	70.6	83.8	3	18	10.5	13.2	-2.7	Aortic and mitral disease. Chronic nephritis.				
16	3010	67	4.9	7.4	70.7	83.4	4	21	11.9	12.7	-0.8	Myocardial weakness; cirrhosis of liver.				
17	2961	69	5.3	7.0*	52.0	70.0	3½	30	16.5	18.0	-1.5	Chronic glomerular nephritis.				
18	2992	48	5.1	7.4	63.9	86.7	5	33	26.1	22.8	+3.3	Cardiovascular disease.				
19	2941	42	5.4	5.9*	32.5	61.4	4	37	33.5	28.9	+4.6	Diabetes with marked ketonuria.				
20	2953	37	6.9	7.3	74.7	104.4	5	38	39.0	29.7	+9.3	Diabetes without ketonuria.				

* Became nauseated and experiment was discontinued.

DISCUSSION.

The results of Table I solve the first of our propositions. There is a fairly definite level of the plasma bicarbonate at which the urine changes its reaction from one more acid than blood to one

more alkaline. In the normal men this occurred when the plasma bicarbonate CO_2 reached 71 ± 5 volume per cent.

To proposition (2), concerning the critical plasma bicarbonate level in pathological cases, the solution is less satisfactory. The critical level was as a rule appreciably higher than in normal men, being in one diabetic 104.4 volume per cent. The other cases ranged from 73.9 to 86.7. Of the nine cases in which sufficient bicarbonate was taken to raise the urinary pH to 7.4, seven showed at this point a plasma bicarbonate higher than the highest critical level shown by any of the normal men, while the other two cases (Experiments 10 and 14) were near the maximum of normal. It is evident that in disease an unusually high concentration of bicarbonate in the blood may be required to turn the urine alkaline. On the other hand, no patient showed an alkaline urine with a plasma bicarbonate below the level of normal men. The practical conclusions indicated by the facts as far as they go are that if less than 0.5 gm. of bicarbonate per kilo body weight turns the urine alkaline no acidosis exists, but *positive* retention tests for acidosis are less decisive. No. 20 (diabetic) as an extreme example, had before the test not only a normal but a high normal alkaline reserve (plasma $\text{CO}_2 = 74.7$). Nevertheless because of the failure of his kidneys to secrete alkaline urine when his blood bicarbonate reached the usual critical level, he received 38 gm. or 1.03 gm. of bicarbonate per kilo, without quite raising his urine to blood alkalinity. No. 18 (cardiovascular disease) received 33 gm. or 0.79 gm. per kilo, before the urine reached blood alkalinity, although his alkaline reserve was at the average normal level (plasma $\text{CO}_2 = 63.9$) at the start. In brief, it appears that when the retention test shows no acidosis, none probably exists; but when it does indicate the presence of acidosis, even our few cases show that the alarm may be false.

The behavior of the kidney to alkaline salts is quite analogous to its disturbed behavior in acid excretion (Henderson and Palmer, 1915, *a, b*), which leads to acid retention.

This phenomenon in connection with the use of sodium bicarbonate as a guide to the grade of acidosis in pathological cases needs further investigation. At the beginning of Experiment 15 the pH of the urine was only 7.0, while the blood plasma CO_2

was 70.6 volume per cent. 18 gm. of NaHCO_3 , however, were necessary to reduce the reaction of the urine to that of normal blood, to pH 7.4. This observation suggests that owing to the impaired kidney function a more acid reaction, for instance, a pH of 7.0, may be utilized in a practical way in these cases. Such utilization would of course be justified only after much more work has been done on this point.

To proposition (3) the answer is definite. The rise in plasma bicarbonate CO_2 per gm. of administered bicarbonate is, except for Experiment 14, as nearly as could be expected equal to the rise calculated on the assumption that the absorbed bicarbonate is uniformly distributed to all the body fluids, the latter being calculated at 700 cc. per kilo of body weight. There are several obvious factors which make the error attending such a calculation necessarily considerable. Variability in absorption from the gastrointestinal tract into the blood stream,¹ distribution among the extravascular fluids, and variation in metabolism (i.e., acid formation during the several hours which may be required for the test). In the normal cases, Experiments 1, 2, 3, 6, and 8 receiving 2, 4, 6, and 10 gm. of sodium bicarbonate respectively, the difference between the calculated and observed CO_2 amounts to less than 2 volume per cent, which is well within the limits of the added experimental errors of the two determinations. The results obtained in Experiments 4, 7, and 9 of Table I show a discrepancy of 3 to 4 per cent between the observed and calculated effects, the difference in each case indicating that only a part of the alkali is present in the blood. Lack of complete absorption of the amount given seems the most plausible explanation in these cases.

Calculation of Bicarbonate Dosage Necessary to Replace Observed Bicarbonate Deficits.—From the above discussion it is quite evident that the therapeutic use of sodium bicarbonate can and should be accurately controlled. Besides causing discomfort of the patient in the form of nausea, vomiting, and even diarrhea by overdosing with bicarbonate, a severe situation may arise from producing as marked a degree of alkalosis (104.4 volume per cent), as observed in Experiment 20. Tileston (1917) re-

¹ Experiments on dogs to study the effect of intravenous sodium bicarbonate on the blood bicarbonate were started, but interrupted by the exigencies of the war.

ported severe tetany in a case of Weil's disease following an intravenous injection of sodium bicarbonate solution, producing a plasma CO_2 of 80 volume per cent. With data at hand it is not possible to say that a high blood bicarbonate is the sole factor in producing tetany or other serious condition, but we may reasonably interpret this finding as evidence that alkalosis very probably does play a part.

For calculating bicarbonate dosage the following table will be convenient.

Weight of individual.		Sodium bicarbonate necessary to raise plasma CO_2 1 volume per cent.
kg.	lbs.	gm.
19	42	0.5
38	84	1.0
57	126	1.5
76	168	2.0
95	210	2.5

In case the individual is obese it would be logical to correct the body weight by estimating and deducting the abnormal weight.

When the organism is forming acid at a rapid rate, as in acute diabetic ketosis, the bicarbonate will raise the plasma CO_2 by less than the calculated amount, because part of the alkali given is neutralized by acids formed during the necessarily gradual (4 to 8 gm. per hour) administration.² Also our results (Table II) indicate that when the dosage is high (over 20 gm.) the effect may fall somewhat short of the calculated because of incomplete absorption. The figures in the table may, therefore be taken as the *minimum* doses that will produce the calculated change in plasma bicarbonate.

Quantitative Relationship between Alkali Retention and Acidosis.—Since the rise caused in volume per cent of plasma bicarbonate CO_2 by absorption of g gm. of sodium carbonate is approximately $\frac{38g}{W}$, and the plasma CO_2 at which urine normally becomes more alkaline than blood averages 71 volume per cent,

²This may have happened in Experiment 14, Table II.

the approximate relationship between plasma CO_2 and bicarbonate retention in the normally reacting body may be expressed by the equation: $\text{Plasma CO}_2 = 71 - \frac{38 g}{W}$.

Such calculation of the plasma CO_2 from the retention test in pathological cases is, however, subject as discussed above to errors from abnormalities in the critical bicarbonate level of the plasma, in unusually rapid formation of acids in the body during the test, and in absorption of the orally administered bicarbonate. These errors in our extreme case (No. 20) amount to so much that they make the retention indicate the plasma CO_2 as 32 volumes per cent, an acidosis of dangerous severity, when as a matter of fact no acidosis existed, the plasma CO_2 being not only normal but a high normal (74.7 per cent plasma CO_2).

However, each of the errors mentioned is such as to make the acidosis estimated from the alkali retention test greater than that actually existing. From this fact and also from the data in our tables, it appears that one may use the above formula if the plasma bicarbonate values calculated are taken only as *minimum values*. It appears, from our results and those of Palmer and Henderson and Sellards, improbable that more severe acidosis often exists than is indicated by the bicarbonate retention test, but it may be indefinitely *less* severe than indicated. With this reservation in mind we may indicate the significance of the bicarbonate retention in terms of acidosis as follows:

Sodium bicarbonate per kilo body wt. required to turn urine alkaline.	Minimum plasma bicarbonate CO_2 indicated.	Maximum degree of acidosis indicated.
gm.	vol. per cent.	
0.0 - 0.5	55	None.
0.5 - 0.8	55 - 40	Mild.
0.8 - 1.1	40 - 30	Moderate to severe.
Over 1.1	Below 30	Severe.

CONCLUSIONS.

1. In normal men the urine becomes more alkaline than the blood ($\text{pH} = 7.4$) when the plasma bicarbonate CO_2 exceeds 71 ± 5 volume per cent.

2. In most of the pathological cases studied the urine did not become more alkaline than blood until a higher plasma bicarbonate had been reached than in normal individuals. Our results show that in pathological conditions there is danger of giving unnecessary and perhaps injurious amounts of bicarbonate if administration is continued until the urine turns alkaline. This fact may explain the disapproval under which the perfectly rational alkali therapy has fallen with some clinicians.

3. Absorbed sodium bicarbonate is distributed in approximate uniformity to the body fluids in general as well as to the blood. The effect of a given dose in raising the plasma bicarbonate may be calculated by assuming that the body contains 700 cc. of fluid per kilo and that the bicarbonate absorbed is distributed therein uniformly.

4. The results indicate the necessity of carefully controlling the therapeutic use of sodium bicarbonate. This may best be done by determination of the plasma bicarbonate. From a preliminary determination the dose necessary to restore the alkaline reserve to normality may be calculated (table on p. 505), while a subsequent determination indicates the actual effect of the administered alkali.

5. As a test for acidosis the alkali retention as used by us (bicarbonate feeding till the urine shows an alkalinity of pH = 7.4) is subject to certain errors, all of which, especially in pathological cases, act to make the results indicate more severe acidosis than exists. The retention test indicates either the approximately correct alkaline reserve, or less. If no acidosis is indicated by the test, its absence can therefore apparently be accepted; but if acidosis is indicated, the finding must be confirmed by blood analysis before accepted.

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STUDIES ON BLOOD SERUM. II

THE INFLUENCE OF PANCREATIC DIGESTION ON THE PROTEOLYTIC ACTIVITY AND THE NON-COLLOIDAL NITROGEN CONTENT.

By WM. H. WELKER AND FREDERICK H. FALLS.

(From the Laboratories of Physiological Chemistry, Experimental Medicine, and Obstetrics, College of Medicine, University of Illinois, Chicago.)

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INTRODUCTION.

Through the work of Delaunay¹ who used Sørensen's formol titration method it became apparent that the amino-acid nitrogen in the blood was increased at the height of digestion. This observation was confirmed by Van Slyke and Meyer,² using the Van Slyke method for the determination of amino-acids. Abderhalden³ states that this increase in amino-acids may be responsible for a positive reaction when his test for pregnancy is applied to the blood serum of an individual at the height of digestion. One⁴ of us found, in working with the Abderhalden reaction, that the inactivated serum control gives a negative result even if the blood is taken at the height of digestion.

This apparent discrepancy, led to a quantitative study of the non-colloidal nitrogen content and the proteolytic activity of the blood serum in the fasting state and at the height of digestion. For this work dogs were selected because of the ease with which relatively large amounts of serum can be obtained from these animals, and because dog serum closely resembles human serum so far as its proteolytic activity is concerned.

¹ Delaunay, Thèse de Bordeaux, 1910.

² Van Slyke, D. D., and Meyer, G. M., *J. Biol. Chem.*, 1912, xii, 399.

³ Abderhalden, E., *Beitr. klin. Infektionskr.*, 1913, i, 243.

⁴ Falls, F. H., *J. Am. Med. Assn.*, 1915, lxv, 524.

EXPERIMENTAL.

Part I.

Apparently normal animals were used for our experiments. The fasting animals had received no food for 24 hours. Those at the height of digestion were killed 8 to 9 hours after the ingestion of a relatively large amount of meat containing a moderate amount of fat. On postmortem examination in the case of the digesting animals absorption was found to be going on rapidly, as evidenced by the injection of the lacteals and the milky appearance of the sera, after centrifugalization of the defibrinated blood. The animals were lightly anesthetized with ether and bled from the femoral artery under aseptic conditions, into a sterile flask containing glass beads. The blood was defibrinated by shaking the flask. It was placed into sterile tubes and immediately centrifugalized under aseptic conditions. The serum was drawn off and combined in a single sterile container to obtain a uniform mixture.

20 cc. samples of the clear serum showing practically no evidence of hemolysis, were placed into three sterile tubes. Into one of the tubes was placed placental tissue prepared according to the Abderhalden¹ technique; *i.e.*, it failed to give off ninhydrin-reacting substances when boiled for 5 minutes with ten times its volume of water. The serum in a second tube was inactivated by heating in a bath at 60°C. for 1 hour. Placental tissue was added at the expiration of this period. The serum in the third tube remained unheated and no placental tissue was added. Into a fourth sterile tube was placed a volume of distilled water equivalent to the volume of serum used in the other three tubes. The usual quantity of placental tissue used in our experiments was added to the water in the tube. This control was used to correct our results for the non-colloidal nitrogen yielded by the placental tissue on incubation. The contents of the tubes were covered with thin layers of toluene. The tubes were then incubated for 48 hours at 37.5°C. in moist chambers. This is more than twice the period of time used in the Abderhalden test. The purpose of this lengthened digestion period was to afford an opportunity for marked increase in the amount of non-colloidal nitrogen where proteolytic activity existed. At the end of this period, a bacteriological examination was made by smears and by cultures on agar. The cultures and smears were negative in all instances.

At the end of the incubation period, 5 cc. of serum were measured into a 250 cc. volumetric flask, 75 cc. of aluminium hydroxide cream were added, and the flask was filled to the mark with distilled water. The contents were well shaken and filtered. 125 cc. of the filtrate, equivalent to 2.5 cc. of serum, were transferred to Kjeldahl flasks and the nitrogen was determined in the usual manner. The contents of the tube containing distilled water and placental tissue were treated in like manner. A total nitrogen determination was made on each of the sera. The results are shown in Table I.

TABLE I.

Dog No.....	Fasting.			At height of digestion.		
	1	2	3	4	5	6
• Total nitrogen.						
	mg.	mg.	mg.	mg.	mg.	mg.
	23.5	24.3	25.2	23.0	21.0	24.0
Non-colloidal nitrogen.						
Serum.....	0.7	0.63	0.78	0.84	0.83	1.16
Serum + placenta.....	0.82	0.62	0.77	0.84	0.99	1.14
Inactivated serum + placenta.....	0.66	0.53	0.66	0.91	0.79	—
Per cent of non-colloidal nitrogen on the basis of total nitrogen.						
	2.98	2.59	3.09	3.65	3.95	4.84
Average per cent of non-colloidal nitrogen.						
	2.88			4.15		

These figures were obtained from a volume of 2.5 cc. of serum.

DISCUSSION.

It will be seen that the blood sera of the animals at the height of digestion, in all cases, contained more non-colloidal nitrogen than the blood sera of the animals in the fasting state. The differences, however, were relatively small. On the basis of the total nitrogen an average increase of a little more than 1 per cent was found. In only two cases (Dog 1, fasting, and Dog 5, digesting) was the non-colloidal nitrogen present in the serum after incubation with placental tissue greater than that in the serum after incubation by itself. This increase was very slight. These results cast doubt on the idea that proteolytic enzymes are swept into the blood stream when rapid absorption takes place at the height of digestion, unless the unsaturated fatty acids⁵ absorbed simultaneously prevent their action.

The results on the inactivated serum and placenta seem to indicate that some of the non-colloidal nitrogen of the serum combines with the serum colloids during the heating at 60°C. This method of controlling the Abderhalden reaction may therefore be open to criticism.

⁵ Jobling, J. W., and Petersen, W. F., *Z. Immunitätsforsch., Orig.*, 1915, **xxiii**, 71.

Part II.

In a second series of experiments, blood serum was obtained from four dogs at the height of digestion as previously described. The total nitrogen of the serum was determined directly. Placental tissue prepared according to the Abderhalden technique was added to some of the serum. This was incubated at 37.5°C. under toluene in a moist chamber for 48 hours. Typical Abderhalden tests were carried out on 3 cc. samples of serum. This is three times the quantity of serum ordinarily employed in the Abderhalden test. The incubation period was twice the length of that usually employed. Serum that had been heated to 60°C. for 2 hours (inactivated) plus placenta, and serum alone were used as controls. These tests were performed in duplicate. After an incubation at 37.5°C. for 48 hours, 10 cc. of the dialysates were actively boiled with 0.2 cc. of a 1 per cent ninhydrin solution for 1 minute.

The strongest ninhydrin reactions were obtained in the tests on the active serum plus placenta. Those obtained in the plain serum control were next in the depth of color obtained. The least intense reactions were noted in the inactivated serum plus placenta controls. The non-colloidal nitrogen was determined in the serum which had been incubated with placental tissue for 48 hours at 37.5°C. in a moist chamber, under toluene. The analytical results are shown in Table II.

TABLE II.

Dog No.....	1	2	3	4
Total nitrogen.				
	mg. 20.45	mg. 22.30	mg. 23.1	mg. 27.6
Non-colloidal nitrogen.				
	0.84	1.05	0.84	1.26
Non-colloidal nitrogen after incubation with placental tissue.				
	0.84	0.96	0.91	0.96
Per cent of non-colloidal nitrogen of untreated serum on the basis of total nitrogen.				
	4.10	4.30	3.63	3.47

These figures were obtained from a volume of 2.5 cc. of serum.

This series of experiments was planned to magnify the results of any proteolytic activity of the blood serum as determined by the Abderhalden technique.

DISCUSSION.

While the serum from each of the four dogs gave indication of a positive Abderhalden reaction, in only one case, that of Dog 3, was there any increase in the non-colloidal nitrogen of the serum after incubation with placental tissue. In two cases there was diminution.

CONCLUSIONS.

1. Under the conditions of our experiments no increase in the proteolytic activity of the serum at the height of digestion was observed.
2. Positive Abderhalden tests obtained at the height of digestion in non-pregnant individuals probably depend on an increase in the amino-acids in the blood serum.
3. The use of inactivated serum as control in the Abderhalden test may be open to criticism.

STUDIES ON BLOOD SERUM. III
THE INFLUENCE OF PREGNANCY ON THE PROTEOLYTIC
ACTIVITY.

BY FREDERICK H. FALLS AND WM. H. WELKER.

(*From the Laboratories of Physiological Chemistry, Experimental Medicine,
and Obstetrics, College of Medicine, University of Illinois, Chicago.*)

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INTRODUCTION.

The proteolytic activity of the blood serum in pregnancy has been extensively investigated in the last few years. Various methods have been used to determine the proteolytic strength of pregnant serum as compared with that of normal serum or of serum obtained from individuals suffering from various pathological conditions.

The dialysis method proposed by Abderhalden is unsatisfactory, for several reasons. First, it is liable to uncontrollable errors from leaks in the parchment thimbles. Secondly, it is a qualitative or at best a very rough quantitative method. Thirdly, the reagent used, ninhydrin, gives characteristic color reactions with substances other than the products of protein digestion and its use is, therefore, open to serious objection as a test for the presence of the latter.

Van Slyke¹ and his collaborators using the Van Slyke method for the determination of amino-acid nitrogen in the blood serum, studied the proteolytic activity of the serum protease in pregnancy and in various pathological conditions. He concludes that by this method, pregnancy cannot be differentiated from other conditions.

¹ Van Slyke, D. D., Vinograd-Villchur, M., and Losee, J. R., *J. Biol. Chem.*, 1915, xxiii, 377. Levin, I., and Van Slyke, D. D., *J. Am. Med. Assn.*, 1915, lxv, 945.

The Fuld-Gross method of antitrypsin determination has been used by Adachi² for the study of this problem, but the difficulty of getting sharp readings renders it unfavorable for any but very rough qualitative work.

The work of Jobling, Eggstein, and Petersen³ using the Folin and Denis method for the determination of changes in the non-coagulable nitrogen content in the blood serum following exposure to various lipoidal adsorbents is important but open to the objection that it is only relatively accurate because of the microchemical methods employed. We undertook the study of this problem using relatively large quantities of serum (20 cc.) and accurate analytical methods.

EXPERIMENTAL.

The dogs were bled to death, under light ether anesthesia, from the femoral artery and the serum was obtained and treated in the manner described in a previous paper.⁴ The non-colloidal nitrogen was determined according to the method⁵ previously described. The dogs used were all healthy and all but one were near term. They were all fasting for at least 12 hours before being used in the experiments to prevent discrepancies in the tryptic titer due to the action of non-specific digestion ferments. That these dogs were not actively digesting was further confirmed by autopsy following the taking of the blood.

The results tabulated are the averages of closely agreeing duplicates.

² Adachi, S., *Z. Geburtshlf. u. Gynak.*, 1914, lxxvi, 516.

³ Jobling, J. W., Eggstein, A. A., and Petersen, W., *J. Exp. Med.*, 1915, xxi, 239.

⁴ Welker, W. H., and Falls, F. H., *J. Biol. Chem.*, 1917, xxxii, 509.

⁵ Welker and Falls, *J. Biol. Chem.*, 1916, xxv, 567.

TABLE I.
Pregnant Dogs.

Dog. No.	Near term.				Early.
	1	2	3	4	5
Total nitrogen.					
	mg.	mg.	mg.	mg.	mg.
	26.2	19.1	21.6	17.65	26.6
Non-colloidal nitrogen.					
Serum (incubated).....	0.85	0.60	0.42	0.56	3.58*
Serum + placenta.....	0.87	0.48	0.56	0.98	3.57
Serum (inactivated) + placenta.....	0.36	0.64	0.63	0.98	3.22

These figures were obtained from a volume of 2.5 cc. of serum.

* 72 hours' incubation.

TABLE II.*
Normal Fasting Dogs.

No.....	1	2	3
Total nitrogen.			
	mg.	mg.	mg.
	23.5	24.3	25.2
Non-colloidal nitrogen.			
Serum.....	0.70	0.63	0.78
Serum + placenta.....	0.82	0.62	0.77
Serum (inactivated) + placenta.....	0.66	0.53	0.66

These figures were obtained from a volume of 2.5 cc. of serum.

* Welker and Falls, *J. Biol. Chem.*, 1917, xxxii, 509.

CONCLUSION.

1. The results of these experiments fail to show any constant increase of proteolytic activity in the blood serum of pregnant animals over that in normal fasting animals.

2. They fail to show except in one case any proteolytic activity whatever.

STUDIES ON BLOOD SERUM. IV
THE INFLUENCE OF THE PUERPERIUM ON THE PROTEOLYTIC
ACTIVITY.

BY **FREDERICK H. FALLS AND WM. H. WELKER.**

*(From the Laboratories of Physiological Chemistry, Experimental Medicine,
and Obstetrics, College of Medicine, University of Illinois, Chicago.)*

(Received for publication, September 26, 1917.)

INTRODUCTION.

In the puerperium remarkable physiological changes take place in the breasts and in the uterus. In the former a great increase in the number and activity of the parenchymal cells takes place. These cells are rich in intracellular ferments. The physiological changes going on in the uterus are equally interesting although of entirely different nature. Instead of being proliferatory they are retrogressive. The uterus in woman decreases in size from an organ weighing about 1,000 gm. to one weighing only about 30 gm. This decrease in size is the result of several factors the most important of which is a decrease in the size of the individual fibers due to retrogressive changes in the cytoplasm of the muscle cells. There is in fact an autodigestion of the fibers without destruction of the cells manifested microscopically by the formation of minute fat droplets in the cytoplasm around the nucleus which tend to coalesce and form larger droplets which are in turn extruded into the intercellular spaces and absorbed. This digestion takes place over a period of 6 weeks at the end of which time the uterus is again normal in size. Whether this is a process of increased ferment activity in the cells of the uterus itself or is part of a general ferment increase in the blood, is of considerable theoretical importance.

We have in the puerperium a period in which great cellular activity (development of the breasts and involution of the uterus) is manifested following a period (pregnancy) in which some observers have reported an increased ferment content of the blood serum. The question arises: How is the ferment content of the blood serum affected by the local changes in the breast and uterus and by parturition?

EXPERIMENTAL.

In order to throw some light upon this problem we took a series of dogs post partum and tested the proteolytic activity of the blood serum according to the technique described in previous papers.¹ The time elapsing following the birth of the pups varied from a few hours to several days. The involution period is much shorter in dogs than in the human species but the loss of weight of the uterus per kilo of weight of the dog is equally great, thus making results comparable. An autopsy was done in each case immediately after the blood was obtained, and the degree of involution of the uterus was determined. The dogs were all fasting for at least 12 hours previous to the time the blood was obtained thus assuring that any increase in the proteolytic content of the serum was not occasioned by non-specific digestion ferment. Milk is present in the breasts of dogs at the time of the birth of the pups or appears very shortly afterward. They differ in this respect from women in whom milk does not appear normally till the 3rd day. Therefore any influence that lactation might have on the ferment activity of the blood serum would be manifest under the conditions of the experiment.

The results tabulated are the averages of closely agreeing duplicates.

Puerperal Dogs.

Dog No.....	1	2	3	4	5	6
Time, post partum.....	3 hrs.	2 days.	3 days.	4 days.	5 days.	7 days.
Total nitrogen.						
	mg.	mg.	mg.	mg.	mg.	mg.
	21.35	18.85	27.20	23.30	16.43	22.84
Non-colloidal nitrogen.						
Serum (incubated).....	0.51	3.50*	1.89	0.63	0.84	0.35
Serum + placenta.....	0.84	3.59	1.70	1.06	0.70	0.25
Serum (inactivated) + placenta..	0.53	2.80	1.61	0.63	0.96	0.28

These figures were obtained from a volume of 2.5 cc. of serum.

*72 hours' incubation.

CONCLUSION.

The results of these experiments fail to show a constant increase in the proteolytic activity of the blood serum in the puerperium. It would therefore appear that the ferment activity in the uterus and the mammary glands does not affect the ferment activity of the blood serum.

¹ Welker, W. H., and Falls, F. H., *J. Biol. Chem.*, 1916, xxv, 567; 1917, xxxii, 509.

STUDIES ON BLOOD SERUM. V

PROTEOLYTIC ACTIVITY AS AFFECTED BY "SO CALLED ANTITRYPTIC ADSORBENTS."

BY WM. H. WELKER AND FREDERICK H. FALLS.

(From the Laboratories of Physiological Chemistry, Experimental Medicine,
and Obstetrics, College of Medicine, University of Illinois, Chicago.)

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INTRODUCTION.

The proteolytic activity of the serum ferments undervarying conditions of experiment has been the subject of a great deal of investigation in the last few years. Investigators have approached the problem from various angles and have used different methods. It is not surprising that in a field of study which is being so rapidly developed under these conditions that divergent views concerning the fundamental principles of the observed phenomena should arise.

The great impetus to this study given by Abderhalden following his announcement of the absolute specificity of the ferments mobilized in pregnancy against chorionic tissue, the so called *Abwehrfermente*, led to an intensive study of the proteolytic power of the blood serum during pregnancy and under various other conditions.

Very soon other views began to be advanced to explain the facts observed in the Abderhalden reaction. Kjaergaard¹ found that any serum allowed to dialyze longer than the 16 to 20 hour period chosen by Abderhalden would give a positive reaction. One of us has shown that blood serum of males and non-pregnant females gave typical positive Abderhalden reactions when human placenta was used as a substrate.²

¹ Kjaergaard, S., *Z. Immunitätsforsch. u. exp. Therap.*, 1914, xxii, 31.

² Falls, F. H., *J. Infect. Dis.*, 1915, xvi, 466.

De Waele³ was probably the first to point out that the placenta was not digested during the Abderhalden reaction but that it acted as an adsorbent for the serum antitrypsin, thus leaving the trypsin free to digest the serum globulins. It was the products of this digestion that dialyzed and gave the positive reaction on boiling with ninhydrin. Jobling, Eggstein, and Petersen⁴ supported this view and showed that the placenta gained nitrogen rather than lost it during the reaction.

Hence it will be seen that two widely divergent views regarding the mechanism of the reaction are held. It was with an idea of contributing to the knowledge regarding these points that the following series of experiments were undertaken.

EXPERIMENTAL.

Fresh blood serum from four series of dogs, fasting, actively digesting, pregnant and post partum, was exposed to various substances under the same conditions for 48 hours to determine the amount of proteolytic activity.

The technique followed is the same as that described in a former paper.⁵

The dogs used were all healthy at the time of bleeding as nearly as could be determined by clinical signs and symptoms, and this was further corroborated by autopsy findings immediately after the death of the animals.

The ether anaesthesia was very light in most cases in order to obviate any possible effect of this anaesthetic upon the proteolytic activity of the blood serum. Numerous bacteriological examinations of the incubated sera showed that by our method of handling the blood, bacterial contamination is practically excluded as a factor. In addition to placenta, however, as used in that series we used kaolin, collodion, and a special laboratory preparation of washed potato starch.

The kaolin was heated in a steam sterilizer for 2 hours, 20 pounds' pressure, before using and about 0.5 gm. was used per

³ De Waele, H., *Z. Immunitätsforsch. u. exp. Therap.*, 1914, xxii, 170.

⁴ Jobling, J. W., Eggstein, A. A., and Petersen, W., *J. Exp. Med.*, 1915, xxi, 239.

⁵ Welker, W. H., and Falls, F. H., *J. Biol. Chem.*, 1917, xxxii, 509.

20 cc. of serum. It was introduced under sterile precautions and then shaken vigorously to break up clumps so as to obtain the maximum surface exposure. Very finely divided washed potato starch, not sterilized, was used in a similar manner. Collodion was used in the form of very thin strips. In each case the serum was covered with toluene after the addition of the various substances. The results are shown in Tables I to IV. These figures are averages of closely agreeing duplicates.

Except in the instances in which a longer period is specifically indicated, 48 hours is the incubation period.

TABLE I.
Analytical Results. (Fasting Dogs.)

Dog No.....	1	2	3	4	5
	mg.	mg.	mg.	mg.	mg.
Total nitrogen....	23.5	24.3	25.2	25.3	24.4
Non-colloidal nitrogen.					
Serum.....	0.70	0.63	0.78	0.77	0.64
Serum + placenta.....	0.82	0.62	0.77	0.77	0.64
Inactivated serum + placenta.....	0.66	0.53	0.66	0.64	0.77
Serum + starch.....	—	0.70	0.91	0.70	0.77
Serum + collodion.....	0.70	0.63	0.77	0.91	0.64
Serum + kaolin.....	0.70	0.63	0.84	0.91	0.64

These figures are for a volume of 2.5 cc. of serum.

TABLE II.
Analytical Results. (Digesting Dogs.)

Dog No.....	1	2	3
	mg.	mg.	mg.
Total nitrogen.....	23.0	21.0	24.0
Non-colloidal nitrogen.			
Serum.....	0.84	0.83	1.16
Serum + placenta.....	0.84	0.99	1.14
Inactivated serum + placenta.....	0.91	0.79	—
Serum + starch.....	0.97	0.89	1.15
Serum + collodion.....	0.70	0.83	1.15
Serum + kaolin.....	1.05	0.89	1.43

These figures are for a volume of 2.5 cc. of serum.

TABLE III.
Analytical Results. (Pregnant Dogs near Term.)

Dog No.....	1	2	3	4	5 ^a (early).
	mg.	mg.	mg.	mg.	mg.
Total nitrogen.....	26.2	19.1	21.6	17.65	26.6
Non-colloidal nitrogen.					
Serum.....	0.85	0.60	0.42	0.56	3.08
Serum + placenta.....	0.87	0.48	0.56	0.98	3.57
Inactivated serum + placenta.....	0.36	0.64	0.63	0.98	3.22
Serum + starch.....	1.23	0.32	0.35	0.84	3.36
Serum + collodion.....	0.81	0.42	0.21	0.77	2.24
Serum + kaolin.....	1.20	0.46	0.35	0.84	3.71

These figures are for a volume of 2.5 cc. of serum.

* 72 hours' incubation.

TABLE IV.
Analytical Results. (Post Partum Dogs.)

Time, post partum.....	4 days.	3 hrs.	2 days.*
	mg.	mg.	mg.
Total nitrogen.....	23.3	21.35	18.85
Non-colloidal nitrogen.			
Serum.....	0.63	0.51	3.50
Serum + placenta.....	1.06	0.84	3.59
Inactivated serum + placenta.....	0.63	0.53	2.80
Serum + starch.....	0.64	0.62	3.42
Serum + collodion.....	0.57	0.60	3.50
Serum + kaolin.....	0.70	0.74	3.50

These figures are for a volume of 2.5 cc. of serum.

* 72 hours' incubation.

DISCUSSION.

Probably the most striking fact that stands out as a result of this investigation is the absolute lack of uniformity in the proteolytic activity of the various sera when exposed to the same substance under identical conditions. The differences observed do not follow any definite rule, but appear to depend rather on the individual differences in the behavior of a given serum toward the various substances.

It is apparent that under the conditions of our experiments proteolytic activity does take place in the serum in the presence of such substances as kaolin, starch, and collodion which could not possibly be digested by proteolytic ferments. Hence it is certainly not necessary to assume that the placenta is digested during an Abderhalden reaction.

It is seen that the higher degrees of proteolytic activity are manifested in the dogs of Tables II, III, and IV. In the fasting series, on the other hand, the proteolytic activity of the blood serum is apparently reduced but still present.

CONCLUSIONS.

1. Substances, non-protein in character, may induce proteolytic activity in blood serum which is equal to or greater than that called forth by placental tissue.

2. The proteolytic activity of serum as called forth by the various substances used in these experiments does not follow any definite rule but shows marked individual variations.

THE INFLUENCE OF CONDITIONS OF BACTERIAL CLEAVAGE OF PROTEINS ON THE CLEAVAGE PRODUCTS.

By TAKAOKI SASAKI.

(From the Laboratory of Medicine, Imperial University of Kyoto, Kyoto, and
the Sasaki Laboratory, Surugadai, Tokyo.)

(Received for publication, September 18, 1917.)

In chronic poisoning caused by assimilation products of bacteria, or the so called alimentary intoxication of children, the variation in bacterial decomposition products according to the change of conditions may be important, although the opinions of authors differ.

In order to investigate the question experimentally, I have studied the products formed from *l*-tyrosine by the action of *proteus vulgaris* and *coli communis*. To the culture fluid I added on the one hand the so called Henderson's phosphate mixture in order to avoid the accumulation of H ion, and on the other hand in the parallel test lactose, in order to further the accumulation of H ion. To both solutions I furthermore added newly precipitated uranyl phosphate, as in my experience the yield of products is increased thereby, the metallic compound apparently acting as a catalysor or activator.

When lactose was added to the culture fluid, I succeeded in isolating *p*-hydroxyphenylethylamine from *l*-tyrosine acted on by *Bacillus coli communis*. When using Henderson's phosphate mixture, I isolated to my surprise *d-p*-hydroxyphenyllactic acid out of *l*-tyrosine by the action of bacteria of the same strain, without finding any trace of the amine. The experiments with *proteus vulgaris* showed exactly the same results.

These experiments demonstrate the fact that from the same substance by the same kind of bacteria, according to the conditions, quite different substances may be produced. In regard to phenylalanine, Amatsu observed the same results, which will be published later.

It cannot be denied that morphologically identical bacteria show individual differences, especially in decomposition products. Nevertheless, the factor referred to above, which has been demonstrated experimentally, can hardly be rejected, especially in the chronic intoxication produced by the poisonous products of bacterial assimilation.

EXPERIMENTAL.

Experiments with proteus vulgaris.

As a standard solution the following culture fluid has been employed:

Sodium chloride.....	5.0 gm.
Monopotassium phosphate.....	2.0 "
Magnesium sulfate.....	0.1 "
Ammonium carbonate.....	1.0 "
Glycerol.....	20.0 cc.
Water.....	1,000.0 "

To this culture fluid has been added, in addition to *l*-tyrosine and uranyl phosphate, Henderson's phosphate mixture in one experiment, and lactose in the other, the experiments otherwise being conducted under exactly the same conditions.

Experiment with the Addition of Henderson's Phosphate Mixture to proteus vulgaris Cultures.

The phosphate mixture was prepared according to the formula of Henderson, by adding to a 20 per cent phosphoric acid solution up to 95 per cent of a normal solution of sodium hydroxide until the neutralizing point was reached as indicated by phenolphthalein. The solution according to Henderson should contain nine parts of disodium phosphate and one part of monosodium phosphate.

We put in a short-necked 1 liter flask with cotton plug, dry sterilized, 800 cc. of culture fluid (650 cc. of standard solution, 150 cc. of Henderson's mixture), together with 0.5 gm. of freshly precipitated, still moist uranyl phosphate and 1.0 gm. of *l*-tyrosine, the optical rotation of which, in 4 per cent hydrochloric acid was $[\alpha]_D^{20} = -12.01^\circ$.

After sterilization in Koch's steam sterilizer, we added to the sterile solution twenty agar cultures of *proteus vulgaris* suspended in the sterile culture fluid. We worked simultaneously with five flasks; i.e., 5 gm. of *l*-tyrosine of *Bacillus proteus* were isolated from decaying pancreas pulp which with the addition of about the same volume of a 2 per cent soda solution had been in the incubator for 24 hours. On the agar surface appeared gray, moist and glittering, easily confluent colonies, from which we could isolate the organisms in pure culture. They proved to be identical in all respects with an authentic laboratory strain of *proteus vulgaris*, according to morphological classification. The bacteria were spread on ordinary agar slants, and cultivated in the incubator for 24 hours; the culture fluid was then added. The bacteria continued to grow well in the mixture, even when in the course of other experiments with lactose the contents of the flasks became almost sterile. Apparently the bacteria were protected from injury from the acids produced by the microorganisms. The mixture was left in the incubator for 10 days, and then for 40 days in a dark cabinet at a temperature of 25-30°.

The following experiments were made at the same time and in the same manner in order to obtain results for comparison. After having tested the purity of the strain of bacteria employed we placed the five portions together in a porcelain evaporating dish and evaporated the mixture in the water bath with the addition of alcohol to syrup consistency, which was then exhaustively and repeatedly extracted with boiling alcohol until no further Millon's reaction could be observed. After evaporation of the alcohol, the residue was dissolved in water. The solution, strongly acidified with phosphoric acid, was then extracted with ether for 24 hours in the Kumagawa-Suto extractor. After evaporation of the ether radial crystal needles were separated. After purification on an earthenware plate, the weight of the crystalline substance was found to be 1.32 gm., and it proved to be nearly pure *p*-hydroxyphenyllactic acid. The optical rotation in aqueous solution was to the right.

For analytical purposes the substance was once more recrystallized from hot water.

0.1342 gm. substance desiccated for 1 day over calcium chloride and dried at 115° lost 0.0066 gm. H₂O.

	Calculated for C ₉ H ₁₀ O ₄ + $\frac{1}{2}$ H ₂ O:	Found:
0.5H ₂ O.....	4.71	4.91

0.1276 gm. substance gave 0.2768 gm. CO₂ and 0.0626 gm. H₂O.

	Calculated for C ₉ H ₁₀ O ₄ :	Found:
C.....	59.31	59.16
H.....	5.53	5.49

For the optical analysis an aqueous solution was taken.

$$[\alpha]_D^{25} = \frac{14.8554 \times 0.25^\circ}{0.2043 \times 1.0036 \times 1} = +18.11^\circ.$$

Contrary to a former experiment we were unable to isolate any *p*-hydroxyphenylpropionic acid.

The residue from the extraction was carefully neutralized with diluted solution of sodium hydroxide to almost neutral reaction and then made strongly alkaline to litmus with saturated sodium carbonate solution. Thereupon the solution was again extracted with ether for 24 hours. There was no demonstrable quantity of *p*-hydroxyphenylethylamine.

Experiment with the Addition of Lactose to proteus vulgaris Culture.

The following were added to the standard solution: 1 gm. of *l*-tyrosine, 1 gm. of lactose, as well as about 0.5 gm. of newly precipitated, still moist uranyl phosphate. Five flasks and 5 gm. of *l*-tyrosine were employed. They were left in the same incubator for the same period as in the preceding experiment. In all other respects the experiment was made exactly as described above. We could obtain no acid extract.

After extraction of the acidified solution, the residue was made strongly alkaline to litmus and was again extracted with ether for about 48 hours. In the receiving flasks, which in the meantime had been changed 3 times, crystals of a slight brownish yellow color were precipitated. The ethereal solution was decanted and shaken with dilute hydrochloric acid. The hydrochloric solution thus separated extracted quantitatively *p*-hydroxyphenylethylamine from the ethereal solution. The crystals remaining

in the receiving flasks were also dissolved in diluted hydrochloric acid. The two hydrochloric solutions mixed together were decolorized with animal carbon. After evaporating, the pure amine hydrochloride was precipitated. The yield amounted to 2.13 gm., melting at 269° (uncorrected).

For analysis the substance was recrystallized with alcohol and was dried at 100° to constant weight.

0.1426 gm. substance neutralized 8.1 cc. 0.1 N NH_3 .

	Calculated for $\text{C}_8\text{H}_{11}\text{ON} \cdot \text{HCl}$:	Found:
N.....	8.09	7.96

The substance was also identified as the chloroplatinate.

0.1728 gm. substance gave 0.0494 gm. Pt.

	Calculated for $(\text{C}_8\text{H}_{11}\text{ON})_2 \text{H}_2\text{PtCl}_6$:	Found:
Pt.....	28.49	28.59

The amine hydrochloride was transformed into the free amine by making the aqueous solution of the hydrochloride alkaline with sodium carbonate, and again extracting with ether.

The substance was dried at 100° to constant weight.

0.1035 gm. substance gave 0.2639 gm. CO_2 and 0.0757 gm. H_2O .

	Calculated for $\text{C}_8\text{H}_{11}\text{ON}$:	Found:
C.....	70.01	69.54
H.....	8.08	8.18

Experiment with the Addition of Henderson's Phosphate Mixture to Bacillus coli communis.

The conditions of the experiment were exactly the same as in the *proteus* experiment. Of the 5 gm. of tyrosine none remained unattacked. We isolated as acid extract a pure substance of about 1.57 gm. melting at 166–168° (uncorrected).

For the analysis the substance was recrystallized from water and melted at 169° (uncorrected).

0.1593 gm. substance desiccated over calcium chloride, weighed and dried at 115° to constant weight, lost 0.0078 gm. H_2O .

	Calculated for $\text{C}_8\text{H}_{10}\text{O}_4 \cdot \frac{1}{2} \text{H}_2\text{O}$:	Found:
0.5 H_2O	4.71	4.90

The rotation was:

$$[\alpha]_D^{25} = \frac{14.6287 \times 0.20^\circ}{0.1483 \times 1.0030 \times 1} = +19.67^\circ$$

0.1242 gm. substance (dried at 115°) gave 0.2685 gm. CO₂ and 0.0602 H₂O.

	Calculated for C ₉ H ₁₁ O ₄ :	Found:
C.....	59.13	58.95
H.....	5.53	5.42

We were unable to obtain any basic extract.

Experiment with the Addition of Lactose to Bacillus coli communis Culture.

The experiment was made in exactly the same way as the second *proteus* experiment. With the addition of lactose the conditions were identical to those in the first experiment. As acid extract 0.131 gm. of crude substance was isolated, which sintered at about 130° and gradually melted between 150 and 162°. The aqueous solution rotated to the right. After having recrystallized from hot water we gained 0.07 gm. of pure substance which melted at 169–170°, and, mixed with *d-p*-hydroxyphenyl-lactic acid (the substance gained and analytically identified in the first experiment), showed no depression of melting point. As basic extract we isolated 0.88 gm. of pure *p*-hydroxyphenylethylamine hydrochloride (268°).

For analysis the substance was recrystallized from alcohol.

The substance was dried at 100° to constant weight.

0.1721 gm. substance neutralized 9.9 cc. 0.1 N NH.

	Calculated for C ₈ H ₁₁ ON · HCl:	Found:
N.....	8.07	8.06

The substance was identified as the chloroplatinate.

0.2615 gm. substance gave 0.0748 gm. Pt.

	Calculated for (C ₈ H ₁₁ ON) ₂ · H ₂ PtCl ₆ :	Found:
Pt.....	28.48	28.60

THE STEREOCHEMISTRY OF THE BACTERIAL DECOMPOSITION OF ALBUMIN.

By TAKAOKI SASAKI AND ICHIRO OTSUKA.

(From the Laboratory of Medicine, Imperial University of Kyoto, Kyoto,
and the Sasaki Laboratory, Surugadai, Tokyo.)

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Walden¹ discovered the fact that when one of the four groups attached to an asymmetric carbon atom is displaced by another, the resulting substance has an optical rotation either in the same or in the opposite direction to that of the parent substance. Since the same substance, for instance, the same amino-acid, may lead to two antipodal chloro- or bromo-acids, depending on the reagent employed for the reaction, it has been justly concluded that in one of the two reactions a change of the original configuration takes place. The mechanism of the inversion at the present state of knowledge remains unexplained. Observations have recently been recorded which seemed to establish the existence of biological reactions analogous to the chemical reactions discovered by Walden.

Sasaki² has observed that *l*-tyrosine gave rise to *d*-*p*-hydroxyphenyllactic acid by the action of *Bacillus proteus*, whereas the *l*-*p*-hydroxyphenyllactic acid was formed by the action of *Bacillus subtilis*.

Subsequently Tsudji,³ working under the direction of Sasaki, discovered that the identical result was obtained when instead of *l*-tyrosine the *dl* form was employed.

Bacillus proteus transformed the *dl*-tyrosine into the *d*-hydroxyacid, whereas *Bacillus subtilis* transformed it into the *l*-hydroxyacid. These observations seemed in harmony with

¹ Walden, P., *Ber. chem. Ges.*, 1896, xxix, 133; 1897, xxx, 2795, 3145; 1899, xxxii, 1833, 1855.

² Sasaki, T., *Acta Scholæ Med. Univ. Imp. Kyoto*, 1916-17, i, 103.

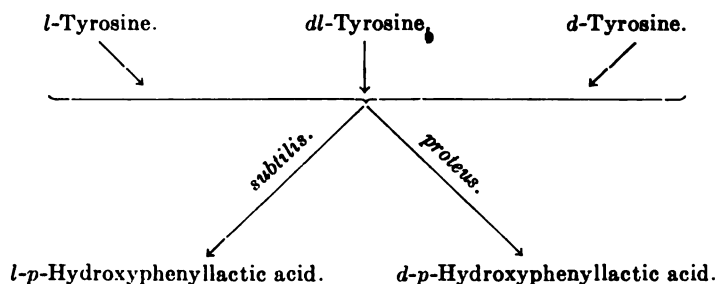
³ Tsudji, M., *Acta Scholæ Med. Univ. Imp. Kyoto*, 1916-17, i, 439.

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those of Walden in which one amino-acid led to different hydroxy-acids, depending on the reagents employed for the intermediary reactions.

A new observation is to be reported in this communication which emphasizes the differences between the two types of reactions. If *l*-tyrosine by a series of chemical reactions is transferred into the *d*-hydroxyacid then *d*-tyrosine under the action of the same set of reagents and under the same conditions will give rise to the *l*-hydroxyacid.

A surprising result was obtained when the experiments with *Bacillus proteus* and *Bacillus subtilis* were performed on *d*-tyrosine. Again *Bacillus proteus* formed the *d*-hydroxyacid and *Bacillus subtilis* the *l*-hydroxyacid. The results are presented in the following diagram:

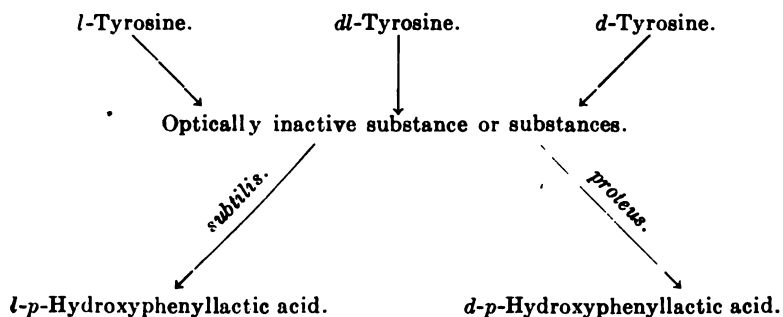


For the explanation of the mechanism of this reaction the choice lies between two hypotheses. One is that the primary hydroxy-acid formed by the microorganism is the *dl* form. One of the antipodes is subsequently consumed by the microorganism, leaving as final product a pure *l* or pure *d* form. This hypothesis may be ruled out on the ground of the observations of Ehrlich and Jacobsen,⁴ who obtained through the action of molds 1.8 gm. of *d*-hydroxyacid from 2.0 gm. of *l*-tyrosine, by those of the present writer who obtained 2.7 gm. of *d*-hydroxyacid from 5.0 gm. of *l*-tyrosine through the action of *Bacillus coli*; i.e., more end-product is obtained than could arise from half of the intermediate product.

The second hypothesis assumes that the intermediate product is a ketone or other intrinsically asymmetric substance, which by

⁴ Ehrlich, F., and Jacobsen, K. A., *Ber. chem. Ges.*, 1911, **xliv**, 888.

the final reaction is converted entirely into one of the two possible asymmetric products. These transformations are presented in the following diagram:



The intermediary product may be assumed to be *p*-hydroxyphenylpyruvic acid. This assumption is in harmony with the views and facts advanced by Neubauer⁵ in connection with the mechanism of oxidation of amino-acids in the animal organism. There are known also other instances of the biological conversion of the symmetric carbonyl groups into an asymmetric alcohol.

EXPERIMENTAL.

Experiments with proteus vulgaris.

The experiments were conducted with the addition of Henderson's phosphate mixture and uranyl phosphate. Five flasks were left in the incubator for 40 days. Of these, one became contaminated with saprophytes, thus leaving for the experiments four, equivalent to 4 gm. of *l*-tyrosine. No pure tyrosine was recovered.

1.99 gm. of crystals in needle form were isolated as an acid extract, which melted at 164–166° after having been purified with kaolin. An aqueous solution turned the sodium light to the right. After a single recrystallization from hot water the substance became quite pure and melted at 169–170°. The output was 1.60 gm.

Thus 1.60 gm. of pure *d-p*-hydroxyphenyllactic acid was

⁵ Neubauer, O., *Deutsch. Arch. klin. Med.*, 1909, xcv, 211. Neubauer, O., and Fromherz, K., *Z. physiol. Chem.*, 1910–11, lxx, 326.

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produced from 4 gm. *L*-tyrosine by the activity of *proteus vulgaris*. 0.1748 gm. substance, desiccated for 1 day over calcium chloride and dried at a temperature of 115° until constant weight was reached, lost 0.0085 gm. H₂O.

	Calculated:	Found:
C ₈ H ₁₀ O ₄ + $\frac{1}{2}$ H ₂ O.....	4.71	4.86

0.1663 gm. substance gave 0.3607 gm. CO₂ and 0.0790 gm. H₂O.

	Calculated for C ₈ H ₁₀ O ₄ :	Found:
C.....	59.32	59.16
H.....	5.53	5.31

The rotation in aqueous solution gave:

$$[\alpha]_D^{20} = \frac{14.6071 \times 0.31^\circ}{0.2451 \times 1.0048 \times 1} = +18.39^\circ \text{ (water).}$$

p-Hydroxyphenylpropionic acid could not be isolated. Nothing could be obtained from the basic extract.

Experiments with Bacillus coli communis.

The experiments were conducted in the same manner as those with *Bacillus proteus*; that is, with the addition of Henderson's phosphate mixture and uranyl phosphate. The strain of *Bacillus coli* used was the same as that employed in our other experiments which have already been reported.

Five flasks, each containing 1 gm. of *L*-tyrosine and twenty agar cultures, remained bacteriologically pure to the end of the experiments, after having been in the incubator for 40 days. The mixture remained alkaline to litmus.

After purification and evaporation of the ether, we isolated an acid extract of 3.80 gm. in crystal needles, as follows:

- I. 3.65 gm. melting at about 150–160°.
- II. 0.155 “ “ “ “ 152–155°.

After recrystallization from hot water with the addition of animal charcoal, 2.56 gm. of pure substance were isolated from the first fraction. The second fraction was recrystallized without the addition of animal charcoal. It yielded 0.15 gm. of pure substance, the melting point being 169–170°. The aqueous solution rotated the sodium light to the right.

For the purpose of analysis the aqueous solution of the substance was extracted once more with ether, in order to free it from animal charcoal. The residue after evaporation of the ether was again recrystallized from hot water.

I. 0.1345 gm. substance gave 0.0068 gm. H₂O.
 II. 0.1259 " " " 0.0062 " H₂O.

	Calculated:	Found:	
		I	II
C ₈ H ₁₀ O ₄ + $\frac{1}{2}$ H ₂ O.....	4.71	5.06	4.92

I. 0.1277 gm. substance gave 0.2761 gm. CO₂ and 0.0621 gm. H₂O.
 II. 0.1197 " " " 0.2592 " CO₂ " 0.0565 " H₂O.

	Calculated for C ₈ H ₁₀ O ₄ :	Found:	
		I	II
C.....	59.32	58.97	59.06
H.....	5.53	5.44	5.28

The rotation in aqueous solution was:

$$[\alpha]_D^{25} = \frac{13.8580 \times 0.29^\circ}{0.2020 \times 1.0046 \times 1} = +19.82^\circ$$

Experiments with Bacillus subtilis.

The spore-bearing bacilli which we isolated according to the method of Roberts and Buchner and identified bacteriologically as belonging to a recognized laboratory stock can be grown readily under a mycodermic surface in the culture fluid containing Henderson's phosphate mixture and uranyl phosphate.

Five flasks each containing 1 gm. *l*-tyrosine and twenty suspensions of growths on agar slants were left in the incubator for 10 days, and then for 40 days in the dark cabinet of a room at 25–30°. All the cultures remained bacteriologically pure. No tyrosine was regained. The remainder of the experiment was conducted in the manner already described.

After evaporation of the ether and purification on a kaolin plate we isolated 0.515 gm. of acid extract in needle crystals. The melting point was about 163–165°. After recrystallization from hot water we isolated 0.35 gm. of the pure substance, melting at 168–169° (uncorrected). The aqueous solution rotated the sodium light to the left. The substance was dried to constant weight at 115°.

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In aqueous solution the rotation was found to be as follows:

$$[\alpha]_D^{25} = \frac{16.1700 \times -0.25^\circ}{0.2057 \times 1.0034 \times 1} = -19.59^\circ$$

0.1569 gm. substance, desiccated for a day over calcium chloride and dried at 115° to constant weight, lost 0.0076 gm. H₂O.

	Calculated:	Found:
C ₂ H ₁₀ O ₄ + $\frac{1}{2}$ H ₂ O	4.71	4.84

0.1493 gm. substance gave 0.3231 gm. CO₂ and 0.0718 gm. H₂O.

	Calculated for C ₂ H ₁₀ O ₄ :	Found:
C.....	59.32	59.02
H.....	5.53	5.38

Nothing could be obtained from the basic extract.

THE CATALASE ACTIVITY OF AMERICAN WHEAT FLOURS.*

By C. H. BAILEY.

(From the Division of Agricultural Biochemistry, Minnesota Agricultural Experiment Station, University Farm, St. Paul.)

(Received for publication, October 23, 1917.)

The researches of Schoenbein on ozone and oxygen transformation paved the way for his discovery (1863) of the power of plant and animal tissues to decompose hydrogen peroxide with the evolution of molecular oxygen. The further study of this phenomenon led to the conclusion that it was occasioned by all enzymes. This view was generally accepted until Loew (1901) showed it to be the result of the activity of a single enzyme, which he called *catalase*. Attention was called by him to its general occurrence in living tissues. There are few, if any, plant tissues which do not show the presence of this enzyme.

Character of the Reaction.

Kastle (1910) concurs with the hypothesis of Bach and Chodat that three types of enzymes are concerned with oxygen transfers in respiration. The *oxidases* are regarded as enzymes having a special aptitude for forming peroxides. These peroxides may be split by *peroxidases*, with the liberation of O, which immediately combines with the oxidizable substance. Or the peroxides, including hydrogen peroxide, may be split into a radical (H₂O, etc.) and molecular oxygen, by the action of *catalase*. The function of catalase, according to Loew (1901), is to destroy the peroxides formed in the living cell which might otherwise prove harmful to it.

The character and constitution of catalase has been the subject of considerable discussion, and several hypotheses concerning it have been advanced. The decomposition of hydrogen peroxide is known to be accelerated by finely divided metals, spongy

* Published with the approval of the Director as Paper 90 of the journal series of the Minnesota Agricultural Experiment Station.

platinum, lead peroxide, manganese dioxide, and other similar inorganic substances. Rammstedt (1910) noted that ignited sea-sand liberated oxygen from hydrogen peroxide. It appears, however, that catalase is an enzyme, since its general properties resemble those of other common enzymes. Its activity is accelerated by a rise in temperature to the extent of 1.5 to 1.7 times per 10°C. A temperature of 70° inactivates it when in a medium containing water, although it is not inactivated in a dry medium by a temperature of 100°. Certain toxic substances, including hydrocyanic acid, hydrogen sulfide, ammonium, sulfocyanide, aminophenol, and metallic nitrates render it inactive.

Catalase in Wheat Flour.

The activity of catalase in the various grades of flour was first discussed by Wender and Lewin (1904). They measured its activity by determining the quantity of oxygen liberated in 1 hour from 35 cc. of hydrogen peroxide solution when mixed with 100 gm. of flour and 200 cc. of water. A high grade flour, No. 0, liberated 64 cc. of oxygen, while a low grade, No. 7½, liberated 246 cc. in 1 hour. It appeared that flour containing the outer and more fibrous portions of the wheat kernel was richer in catalases than that from the inner portion. Wender (1905) later extended this investigation, and in addition to studying the various grades from several different mills, made similar tests on other cereal products. Wheat starch liberated but 8 cc., while wheat bran liberated 342 cc. of oxygen under the same working conditions. The flour grades when tested in a similar manner gave practically the same results as were reported in the earlier paper by Wender and Lewin.

Licchti (1909) found that the quality of a flour can be judged by the quantity of oxygen which it liberated from hydrogen peroxide.

Miller (1909) followed a somewhat different procedure in determining catalase activity. He extracted the flour with water for 4 hours, and added an aliquot of this extract representing 0.5 gm. of flour to a solution of hydrogen peroxide. After 2 hours the residual hydrogen peroxide was determined by titrating with 0.1 N KMnO_4 . The relative catalase activity, stated in terms

of H_2O_2 decomposed, of the different flour grades was found to be quite similar to that reported by Wender.

Rammstedt (1910) published a critique of the methods employed for determining the quality of flour, and called attention to the liberation of oxygen from hydrogen peroxide in the presence of ignited sea-sand.

The writer has found no mention in the American literature on flour qualities to the use of the catalase activity tests in determining the grade of flours, nor, so far as he is aware, has this test been applied extensively for this purpose to American wheat flours. Most of the German articles were published in journals which do not come to the attention of many American cereal chemists and flour technologists. It accordingly appeared advisable to ascertain whether the relation between the rate of the decomposition of hydrogen peroxide and the grade of the flour is as exact in the case of American flours as it proved to be in the Continental investigations cited, and if so, to call the attention of American workers in this field to the usefulness of this test.

Apparatus for Testing Catalase Activity.

The first step in this study was to develop an apparatus for determining the rate of the decomposition of H_2O_2 . The arrangement finally decided upon is shown in Fig. 1. The bottle (A) is of 500 cc. capacity, and is fitted with a rubber stopper. It is half submerged in a water bath which is maintained at a temperature of 20°C . The rubber stopper has three holes, through one of which the tube of a 30 cc. cylindrical separatory funnel with open top (B) is forced. The second opening is provided with a tightly fitting tube with glass stop-cock (C), while the third opening contains the glass delivery tube (D). This delivery tube is connected by a short piece of rubber pressure tubing to the inlet tube of a 100 cc. Hempel-Winkler gas burette (E). All the joints must be tight.

Method for Determining Catalase Activity.

In making the test 1 gm. of flour is placed in a mortar and triturated with about 25 cc. of distilled water. This is poured into the bottle (A), and the mortar washed out with several

portions of water until a total of 100 cc. has been mixed with the flour. The bottle is then tightly stoppered, and the flour and water mixture allowed to attain the temperature of the water thermostat. 5 cc. of a perhydrol (30 per cent H_2O_2) solution, made by mixing one part of perhydrol with four parts of water, are placed in the cylindrical separatory funnel. When the water level in the gas burette is brought to the zero mark, and its cock closed, the stop-cock of the separatory funnel (B) is opened, and by opening the cock of the tube (C) the perhydrol solution is admitted into the flour suspension in the bottle. The contents of the bottle are mixed by rotating for a moment, the cocks of the tube (C) and of the separatory funnel (B) are closed, and the stop-cock of the gas burette is at once opened. The water level in the overflow bulb (F) is kept lower than that in the gas burette in order that no internal pressure may develop in the system and thus unseat the stopper or cocks. After 15 minutes the contents of the bottle (A) are rotated momentarily to wash down light, fibrous material which may have floated and adhered to the sides of the bottle. The bottle is not disturbed again until after the first reading is taken at the end of exactly 30 minutes. It is then rotated as before, and allowed to stand until a total of exactly 60 minutes has elapsed, when the second and final reading is taken. The volume of gas which has replaced water in the gas burette is corrected to a temperature of 20° and a pressure of 760 mm.

Results of Tests of American Wheat Flours.

To ascertain the relation between the catalase activity and the grade of American wheat flours, four series of samples were obtained from as many mills in Minnesota. These were presumably all milled from 1915 crop wheat mixtures in which the northern hard spring wheat predominated. Each series included a sample of Patent flour, three series included a Straight, all included a First Clear, and that from the largest mill included a Second Clear flour. In two instances the percentage of the total edible flour produced which was represented in each of the grades was reported. The mills furnishing these samples were of varying capacity, Mill A being of 5,000 barrels per 24 hours capacity, Mill B of 1,000 barrels, Mill C of 850 barrels, and Mill D of 500 barrels.

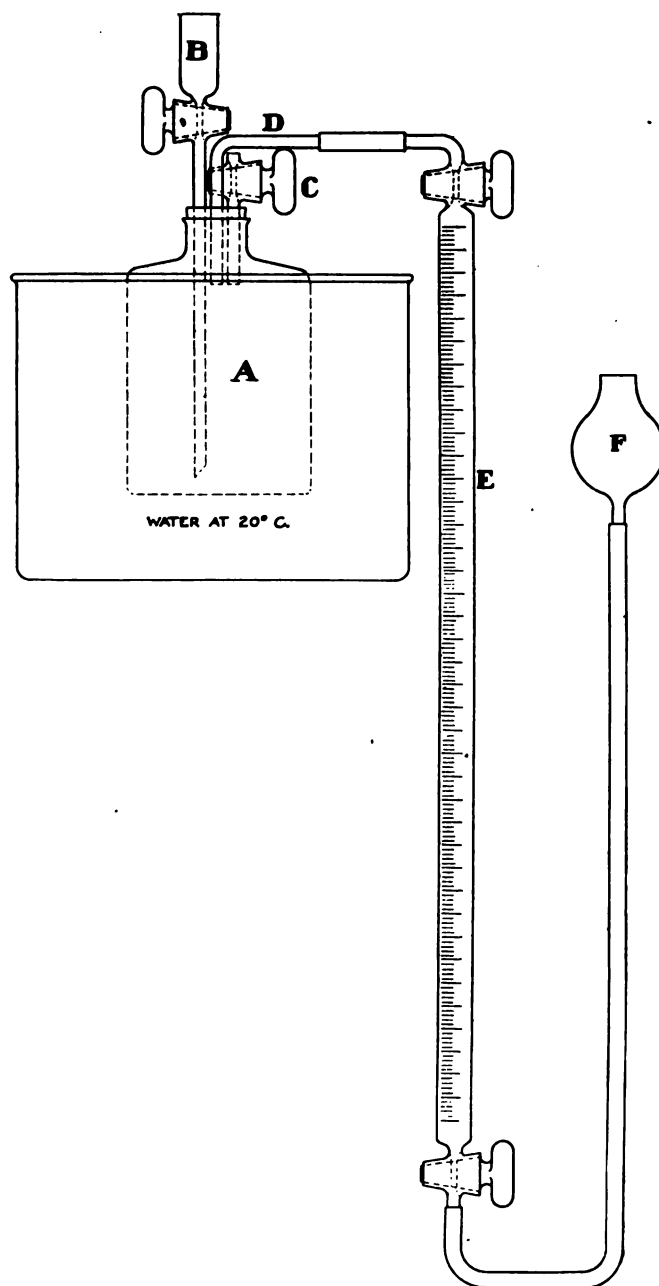


FIG. 1.

In addition to these four series of flours, seven samples of Straight flour containing from 0.45 to 0.52 per cent of ash were tested. These seven samples were produced by as many different mills.

The results of the catalase activity tests of these flours are shown in the table. This table gives the source and grade of the

Catalase Activity Tests of American Wheat Flours, Crop of 1915.

Laboratory No.	Flour grade.	Ash.	Catalase activity. Cc. of O ₂ evolved.	
			In 30 min.	In 60 min.
Mill A, 5,000 barrels capacity.				
B 549	Patent.....	<i>per cent</i> 0.36	6.9	11.6
" 550	1st Clear.....	1.04	41.1	52.4
" 551	2nd "	2.00	58.9	97.2
Mill B, 1,000 barrels capacity.				
B 571	83 per cent Patent.....	0.42	10.2	14.2
" 572	100 " " Straight.....	0.48	16.1	22.9
" 573	17 " " Clear.....	0.72	34.9	45.1
Mill C, 850 barrels capacity.				
B 574	"A" Grade.....	0.49	14.1	20.6
" 575	"L" "	0.54	16.7	26.2
" 576	"T" "	0.93	38.4	51.6
" 577	"E" "	1.42	72.4	80.0
Mill D, 500 barrels capacity.				
B 578	80 per cent Patent.....	0.43	5.5	11.5
" 579	Straight.....	0.48	12.6	19.9
" 580	20 per cent Clear.....	0.86	30.1	43.4
Straight flour from various sources.				
B 525	Straight.....	0.45	5.7	9.1
" 540	"	0.46	6.4	10.7
" 529	"	0.47	6.5	11.0
" 566	"	0.48	6.6	11.5
" 569	"	0.49	6.6	11.6
" 528	"	0.50	8.9	14.6
" 588	"	0.52	12.0	20.2

samples, their ash content in per cent, and their catalase activity expressed in terms of the cc. of O_2 evolved in 30 and 60 minutes with a 1 gm. charge of flour.

It will be observed that the parallelism between the percentage of ash and the quantity of O_2 evolved is not exact; it is remarkably close, however, and shows the test to be of considerable value in indicating the grade of flour. It is hardly to be expected that a determination of enzyme activity could be made with as great precision as that of the percentage of ash or mineral matter. In general we find that with double the ash content the catalase activity is increased to about 340 per cent of the lower value, while treble the ash content accompanies a catalase activity of about 500 per cent of the lower value. The catalase activity thus increases at a more rapid rate than the percentage of ash. This is of distinct advantage in distinguishing between the various grades of flour, and compensates in large part for the diminished accuracy with which such tests can be made. An additional advantage of this procedure over determining the percentage of ash is that it can be made in a shorter time, and with less elaborate and expensive equipment, since it dispenses with the muffle furnace and crucibles of platinum or other material.

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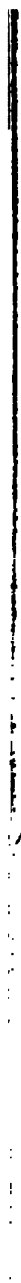
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